ROLE OF THE C-TERMINUS OF PROTEIN KINASE C-RELATED KINASE IN CELL SIGNALLING

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A THESIS SUBMITEED

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

NATIONAL UNIVERISTY OF SINGAPORE

2007

ACKNOWLDEGMENTS

I wish to express my sincere gratitude to Dr. Duan Wei for giving me guidance and advice along the way.

Special thanks to Prof. Halliwell for his patience and giving me a much needed boost for the final lap.

Special appreciation to Bee Jen for her technical help, invaluable advice and support, without which this is not possible.

Sincere thanks to:
Prof. Jey and Dr. Arun for their help.
Samo, Siao Ching, Charmain, Dawn, Kai Ying and Wishva for their friendship and help, scientifically or otherwise.
Tiffany and Vell for being there.

I would also like to acknowledge the Research Scholarship award from the National University of Singapore and grants from the Biomedical Research Council, Singapore.

Last but certainly not least, I would like to dedicate this thesis to my mum for her concern and love throughout my candidature.

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List of Abbreviations

α	Alpha
ACC	Anti-parallel coiled coil
ADAM	A Disintegrin And Metalloprotease
AGC family	Protein Kinase A, Protein Kinase G and Protein Kinase C
aPKC	Atyptical Protein Kinase C
Arg	Arginine
ATP	Adenosine 5' Triphosphate
β	Beta
bp	Base Pair
BSA	Bovine Serum Albumin
^{o}C	Degree Celcius
CaM	Calmodulin
CaMK	Calmodulin Kinase
cAMP	Adenosine 3', 5'- cyclic monophosphate
cDNA	complimentary deoxyribonucleic acid
CDK	Cyclin-dependent kinases
CL	Cardiolipin
CO	carbon monoxide
COS	CV-1 Origin, SV40
cPKC	Conventional Protein Kinase C
C1	Conserved Region 1
DAG	Diacylglycerol
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic Reticulum
ERK	Extracellular signal-regulated protein kinase
FLC	Furin-Like Convertase
GAP	GTPase activating protein
GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GPCR	G Protein Coupled Receptor
GMP	Guanosine Monophosphate
GSK3	Glycogen synthase kinase 3
GST	Glutathione S-Transferase
GTP	Guanosine Triphosphate
h	Hours
Hist	Histidine

HR	Homology Region
IP	Immunoprecipitation
IP_3	Inositol-1,4,5-triphosphate
IPTG	Isopropylthio-β-D-galactosidase
LPA	Lysophosphatidic acid
Lys	Lysine
M	Molar
MAPK	Mitogen Activating Protein Kinase
MBP	Myelin Basic Protein
μ	Micro
ug	Microgram
mg	Milligram
min	Minute(s)
ul	Microlitre
ml	Millilitre
uM	Micromolar
mM	Millimolar
mRNA	Messenger RNA
NGF	Nerve Growth Factor
nPKC	Novel Protein Kinase C
NMR	Nuclear Magnetic Resonance
NO	Nitric oxide
NP-40	Nonidet P-40
OH	Hydroxyl
PA	Phosphatidic acid
PAK	Protease Activated Kinase
PCR	Polymerase Chain Reaction
PDK-1	Phosphoinositide-Dependent Kinase 1
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PiP_2	Phosphatidylinositol-4,5-biphosphate
PiP_3	Phosphatidylinositol-3,4,5-triphosphate
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
PKN	Protein Kinase N
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl Fluoride
PRK	Protein Kinase C-Related Kinase
Pro	Proline
PS	Phosphatidylserine
S	Second(s)

Ser	Serine
SDS	Sodium Dodeyl Sulfate
SDP-PAGE	Sodium Dodely Sulfate- Polyacrylamide Gel Electrophoresis
TAE	Tris, Acetic acid, EDTA
TBE	Tris, Boric acid, EDTA
TEMED	N, N, N', N' tetramethylethylenediamine
Thr	Threonine
TPA	12-O-tetradecanoylphorbol 13-acetate
Tween-20	polyoxy-thyl-sorbitan monolaurate
Tyr	Tyrosine
V5	Variable Region 5
WT	Wild Type
w/v	weight: volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Publications

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1. **Wee Guan Lim**, Yimin Zhu, Chern-Hoe Wang, Bee Jen Tan, Jeffrey Armstrong, Terje Dokland , Hongyuan Yang, Yi-Zhun Zhu, Tian Seng Teo and Wei Duan, "The last five amino acid residues at the C-terminus of PRK1/PKN is essential for full lipid responsiveness"**,** ***Cellular Signalling,*** 17:1084-109*7,* 2005. (Impact Factor 5.185).

2. Yimin Zhu, **Wee Guan Lim,** Bee Jen Tan, Tian Seng Teo & Wei Duan, "Identification of an integral plasma membrane pool of protein kinase C in mammalian tissues and cells", ***Cellular Signalling,*** 17:1125-1136, *2005.* (Impact Factor 5.185).

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5. **Wee Guan Lim**, Bee Jen Tan, Yimin Zhu, Shufeng Zhou, Jeffrey S. Armstrong, Qiu Tian Li, Qihan Dong, Eli Chan, Derek Smith, Chandra Verma, Seng-Lai Tan and Wei Duan, "The Very C-Terminus Of Prk1/Pkn Is Essential For Its Activation By RhoA And Downstream Signaling", ,***Cellular Signalling***. Available online 19 January 2006 (Impact factor: 4.741).

6. Sui Sum Yeong, Yimin Zhu, Derek Smith, Chandra Verma, **Wee Guan Lim**, Bee Jen Tan, Jeffery S. Armstrong, Shufeng Zhou and Wei Duan, "The last ten amino acids beyond the hydrophobic motif are critical for the catalytic competence and function of Protein Kinase C α", Journal of Biological Chemistry, 2006 Oct 13;281(41): 30768-81.

Summary

Protein Kinase C-related kinases (PRK) are members of the protein kinase C (PKC) superfamily of serine/threonine protein kinases. The structure of members of the PKC superfamily is highly conserved, with an N-terminal regulator domain linked to a C-terminal catalytic domain via a linker segment. The catalytic core of all PKC superfamily members has 40-50% sequence identity. At the end of the catalytic domain, there is a C-terminal tail consisting of approximately 70 amino acid residues called the variable region 5 (V5) domain. This V5 domain is present in all PKC/PRKs, ranging from yeast to humans, but with a much lower sequence homology compared with that of the catalytic core.

This project aims to determine the V5 domain as a determinant in isozyme-specific regulation in PRK1 and PRK2. The V5 region appears attractive for specific regulation due to its great variability in terms of amino acid length and sequence among the isozymes in the PKC family.

Both PRK1 and PRK2 tolerated removal of up to seven amino acids from the C-terminal (PRK1-Δ940 and PRK2-Δ978), where the hydrophobic motif was deleted, thus it appears that the hydrophobic motif is dispensable for PRK1 and PRK2 activity. However, PRK1 and PRK2 lacking the eight amino acids or more were catalytically inactive. Co-immunoprecipitation studies indicate that the absence of the hydrophobic motif in PRK1 and PRK2 did not affect PDK-1 binding. Thus, the general proposal that the hydrophobic motif functions as a substrate docking site for PDK-1 may not be accurate. The relation between PDK-1 binding and the consequent phosphorylation of the activation loop in PRK1 and PRK2 and kinase activity demonstrated that the

interaction of PDK-1 with PRK1 and PRK2, and the productive phosphorylations of the activation loop are separate biochemical events. Activation studies indicate this C-terminus segment of residues is necessary for optimal activation.

We propose that the importance of the very C-terminus in conferring the catalytic competence in PRK1 and PRK2 may be a common feature among several other PKC isozymes. This study has identified the extreme C-terminal of seven amino acids in PRK2 is critical for the function and regulation of the kinase. The data suggest that this segment of residues in the V5 domain in necessary to maintain critical interaction with the kinase domain to allow proper folding for catalysis.

Chapter 1: Introduction

1.1 Signal Transduction

Signal transduction at the cellular level refers to the process of converting one kind of signal or stimulus from the outside of the cell, to another signal inside the cell. In endocrine signaling, signaling molecules, called hormones, act on target cells distant from their site of synthesis (by cells of endocrine organs). By contrast, in autocrine signaling, cells respond to substances that they themselves release. Signal transduction involves a sequence of biochemical events inside the cell, which take place in a timeframe as fast as milliseconds to as long as days. In signal transduction processes, multiple enzymes and other molecules become engaged in the events that proceed from the initial stimulus. In such cases, the chain of steps is referred to as a "signaling cascade" or a "second messenger pathway" and enables a stimulus to elicit a large response. The eventual outcome is an alteration in cellular activity and changes in gene expression in the responding cells.

1.1.1 Signal Receptors

A cell will only react to a signal if it has a receptor that recognizes that specific signal. Receptors usually have high specificity for a specific signal. This specificity is directly related to the molecular contours of the receptor and the signal, so that they fit each other at the molecular level. Once bound, the receptor will transmit the signal into the cell. It is noteworthy that signal receptors may be localized in different compartments in cells, and even the same receptors within the same cell may have different biological functions when situated in different locations.

1.1.1.1 Cell surface receptors

Fat-soluble signals, such as hormones and some vitamins, are thought to simply diffuse across the membrane. Their receptor proteins are usually found within the cell. In contrast, most chemical signals are water-soluble and thus unable to cross the lipid bilayer of the cell membrane. Their receptor proteins must therefore span the membrane. Although cell-surface receptors differ in the way that they transmit information into the interior of the cell, in mammalian cells most can be generalized into three distinct and large families based on the mechanism they use to accomplish this transmission: ion-channel-linked receptors, G-protein-linked receptors, and enzyme-linked receptors [1].

1.1.1.2 Ion-channel-linked receptors

These are fast acting receptors, exemplified by the nervous system that allows sub-millisecond transmission times across synapses. The receptors for some transmitters themselves are ion channels. Alternatively, the receptors can link with ion channels. Chemical signals in the form of neurotransmitters are transduced by ion-channel-linked receptors directly into an electrical signal in the form of a voltage difference across the plasma membrane. This occurs when a neurotransmitter binds to this type of receptor, altering its conformation to open or close a channel (often through or near the receptor) to the flow of Na^+, K^+, Ca^{2+}, or Cl^- ions across the membrane. With the change of flow of ions, the potential of target cells subsequently changes, thus signals can be transduced by ion-channel-linked receptors [2].

1.1.1.3 G-protein-linked receptors

Many hormones, neurotransmitters and sensory stimuli elicit cellular responses through the targeted activation of cell surface receptors coupled to Gq family G proteins. The Gq family members, Gq, G11, G14, and G15/16, like all heterotrimeric G proteins, are composed of three subunits, Gα, Gβ and Gγ, that cycle between inactive and active signaling states in response to guanine nucleotides [3, 4]. These membrane-bound proteins are engaged and activated by G protein-coupled receptors (GPCRs) for the GTP-dependent transduction of extracellular signals into cellular responses. The receptor-bound conformation of the G protein favors the displacement of GDP by GTP on the Gα subunit and induces dissociation of the heterotrimer subunits from the receptor and from each other. Gα-GTP and the Gβγ dimer then transmit the receptor-generated signals to downstream effector molecules and protein binding partners until the intrinsic GTPase activity of Gα hydrolyzes GTP to GDP and the inactive subunits reassociate.

The canonical effector molecules of activated, GTP-bound Gq family Gα subunits are the β-isoforms of phospholipase C (PLC-β). Gqα, G11α, G14α, and G15/16α (mouse/human orthologues, respectively) bind and stimulate PLC-β enzymes to initiate inositol lipid signaling. PLC-β enzymes catalyze the hydrolysis of the minor membrane phospholipid phosphatidylinositol bisphosphate, PIP_2, to release inositol trisphosphate (IP_3) and diacylglycerol (DAG) [5]. These second messengers serve to propagate and amplify the Gα-mediated signal with calcium mobilization following release from IP_3-regulated intracellular stores and DAG-mediated stimulation of protein kinase C (PKC) [6, 7]. Inositol lipids, DAG, PKC and calcium each participate in multiple signaling networks and in this way link Gqα family members to a host of different cellular events [8].

There also are many reports of signaling pathways and global cellular responses activated by Gqα family proteins in which the mediating Gα-effector protein(s) are unknown. Signaling downstream of these Gα involves diverse and complex kinase cascades that ultimately lead to regulated gene transcription and changes in cell physiology. Each Gqα family member has been implicated in regulating one or more of the mitogen-activated protein kinase (MAPK) pathways in cultured cells, although the precise mechanisms of signal transfer are not well understood [9-15]. In some instances, PKC is the critical component linking Gqα signaling to activation of MAPK pathways [16].

1.1.1.4 Enzyme-linked receptors

There are at least five classes of enzyme-linked receptors;

(1) receptor tyrosine kinases,

(2) tyrosine kinase-associated receptors,

(3) receptor serine/threonine kinases,

(4) transmembrane guanylyl cyclases, and

(5) histidine-kinase-associated receptors.

The first two classes are the most abundant in cells. Enzyme-linked receptors are single-pass transmembrane proteins with an extracellular ligand-binding domain and an intracellular catalytic or enzyme-binding domain. The great majorities of receptors are protein kinases or are associated with kinases. Binding of agonists to receptors induces a conformational change of the receptors. Such conformational changes lead to the activation of kinases that are either intrinsic to the receptor or associated with the receptor. Thus the signals are transduced from the extracellular to the intracellular environment [17, 18].

Besides the five groups of cell surface receptors, there are some other cell surface receptors that do not fit into these classes. There is one group of cell surface receptors that activate the signaling pathways depending on proteolysis. For example, the receptor protein Notch is activated by cleavage. In vertebrates, most Notch protein is targeted to the cell surface after processing by a furin-like convertase (FLC) at cleavage site 1 [19, 20]. The S1 cleavage divides Notch into two polypeptides: one contains almost the entire extracellular domain and the other contains a short ectodomain and a long cytoplasmic tail. After ligand binding, a change occurs that renders the region just proximal to the membrane susceptible to cleavage by metalloproteases of the ADAM family (a disintegrin and metalloprotease), especially at a position 12 amino acids from the membrane. This site is referred to as the S2 site. After ligand-induced ectodomain shedding, Notch undergoes cleavage at a third site (S3) located within the transmembrane domain by the enzyme γ-secretase. The freed intracellular domain enters the nucleus, where it switches a DNA-bound corepressor complex into an activating complex, leading to activation of selected target genes [21, 22].

1.1.1.5 Nuclear receptors

The nuclear receptors are localized in the cytosol and/or nucleus. Many of the natural ligands of nuclear receptors are lipophilic hormones that enter the cell in a passive manner or by active transport mechanisms. These agonists include steroid hormones, thyroid hormones, retinoids, and vitamin D. The ligand binding activates the transcriptional regulation function of the receptor by binding to specific DNA sequences adjacent to the target genes. The receptors are structurally related and are part of the nuclear receptor family. Some receptors that are activated by intracellular

metabolites are also included in this family. Some nuclear receptors, such as those for cortisol, are located in the cytoplasm, and translocate to the nucleus after ligand binding, while other nuclear receptors, such as those for thyroid hormone and retinoids, are bound to DNA in the absence of ligands. The cognate receptors are quite promiscuous with respect to the nature of the ligand. The binding of hormones to the receptors allows the inhibitory protein complexes which are bound to the inactive receptors to dissociate while the binding of coactivator protein to the receptors induces gene transcription [23].

1.1.1.6 Intracellular enzymes as receptors

Nitric oxide (NO) and carbon monoxide (CO) can rapidly diffuse across the membrane and bind to iron in the active site of guanylyl cyclase, thereby stimulating this enzyme to produce the small intracellular mediator cyclic GMP. The cyclic GMP can induce responses in target cells, for example, keeping blood vessels relaxed [24].

1.1.2 Intracellular signaling molecules

After extracellular signaling molecules bind to the receptors, the signals are relayed into the cell interior by a combination of small and large intracellular signaling molecules. The former is second messengers; the latter are called intracellular signaling proteins and includes G proteins, small GTPase and protein kinases [25-27].

1.2 Signal transduction by phosphorylation

A central tool for signal transduction in a cell is phosphorylation. Protein phosphorylation is a ubiquitous regulatory mechanism in both eukaryotes and prokaryotes. Intracellular phosphorylation by protein kinases, triggered in response to

extracellular signals, provides a mechanism for the cell to switch on or off many diverse processes. These processes include metabolic pathways, kinase cascade activation, membrane transport and gene transcription. The reverse reaction of dephosphorylation is catalysed by protein phosphatases that are controlled by response to either the same or different stimuli so that phosphorylation and dephosphorylation are delicately controlled to achieve optimal state of phosphorylation at a given time. In eukaryotes, the protein kinase domains responsible for phosphorylation on serine, threonine, or tyrosine residues are the first, second and third most common domains in the genome sequences of yeast (*S. cerevisiae*), the worm (*C. elegans*), and the fly (*D. melanogaster*), respectively, indicating the importance of phosphorylation signaling in higher organisms [28]. The human genome contains 575 eukaryotic protein kinase domains which represents 2% of the total genome [29]. In prokaryotes, signaling by phosphorylation is equally important. In *E. coli*, there are 62 genes that encode proteins involve in dual histidine/response regulator systems, representing approximately 1.5% of the entire genome.

1.2.1 History and definition

The first protein kinase obtained in a purified form was the Ser/Thr-specific phosphorylase kinase of muscle in 1959 [30]. With the discovery of the Tyr-specific protein kinases [31], the Ser/Thr-specific protein kinases were joined by another extensive class of protein kinases of regulatory importance, to which a central function in growth and differentiation processes was soon attributed. At present, several hundred different protein kinases are known in mammals, most of which are

Ser/Thr or Tyr-specific. In addition, there are some protein kinases that phosphorylate other amino acids.

Based on the nature of the acceptor amino acids, four classes of protein kinases can be distinguished [32]:

- Ser/Thr-specific protein kinases esterify a phosphate residue with the alcohol group of Ser or Thr residues.
- Tyr-specific protein kinases create a phosphate ester with the phenolic OH group of Tyr residues.
- Histidine-specific protein kinases form a phosphorous amide with the 1 or 3 position of His. The members of this enzyme family also phosphorylate Lys and Arg residues.
- Aspartate- or glutamate-specific protein kinases create a mixed phosphate-carboxylate anhydride.

Reversible phosphorylation of proteins on Ser/Thr and Tyr residues functions as a switch in signaling pathways. The phosphate esters formed on proteins by the action of protein kinases are stable modifications that cause profound changes in the activity of cellular proteins. Because of the stability of the phosphate esters, protein phosphatases are required for their removal. The concerted and highly regulated action of both protein kinases and protein phosphatases is used by the cell to create a temporally and spatially restricted signal influencing the activity state of proteins in a highly specific way.

1.2.2 Classification of superfamily of protein kinase

Protein kinases are classified according to the scheme proposed by Hanks and Hunter [32] based on similarity in catalytic domain amino acid sequence. Using this classification has the advantage of revealing other features held in common by the different members of a family. Therefore kinases having catalytic domains with high homology are likely to:

1) be similar in overall structural topology,

2) have similar modes of regulation, and

3) have similar substrate specificities.

The subgroups are:

1. **AGC Group**
    1. AGC Group I
       Cyclic nucleotide regulated protein kinase (PKA & PKG) family
    2. AGC Group II
       Diacylglycerol-activated/phospholipid-dependent protein kinase C (PKC) family
    3. AGC Group III
       Related to PKA and PKC (RAC/Akt) protein kinase family
    4. AGC Group IV
       Kinases that phoshorylate G protein-coupled receptors family
    5. AGC Group V
       Budding yeast AGC-related protein kinase family
    6. AGC group VI
       Kinases that phosphorylate ribosomal protein S6 family
    7. AGC Group VII
       Budding yeast DBF2/20 family
    8. AGC Group VIII
       Flowering plant PVPK1 protein kinase homolog family
    9. AGC Group Other
       Other AGC related kinase families
2. **CaMK Group**
    1. CaMK Group I
       Kinases regulated by Ca^{2+}/CaM and close relatives family
    2. CaMK Group II
       KIN1/SNF1/Nim1 family

3. CaMK Other
   Other CaMK related kinase families

3. **CMGC Group**

   1. CMGC Group I
      Cyclin-dependent kinases (CDKs) and close relatives family
   2. CMGC Group II
      ERK (MAP) kinase family
   3. CMGC Group III
      Glycogen synthase kinase 3 (GSK3) family
   4. CMGC Group IV
      Casein kinase II family
   5. CMGC Group V
      Clk family
   6. CMGC Group Other

4. **PTK Group** - `Conventional' protein-tyrosine kinases

   Group I-IX Non-membrane spanning protein-tyrosine kinases

   Group IX-XXI membrane spanning protein-tyrosine kinases

   1. PTK group I
      Src family
   2. PTK Group II
      Tec/Atk family
   3. PTK Group III
      Csk family
   4. PTK Group IV
      Fes (Fps) family
   5. PTK Group V
      Abl family
   6. PTK Group VI
      Syk/ZAP70 family
   7. PTK Group VII
      Tyk2/Jak1 family
   8. PTK Group VIII
      Ack family
   9. PTK Group IX
      Focal adhesion kinase (Fak) family
   10. PTK Group X
       Epidermal growth factor receptor family
   11. PTK Group XI
       Eph/Elk/Eck receptor family
   12. PTK Group XII
       Axl family
   13. PTK Group XIII
       Tie/Tek family
   14. PTK Group XIV
       Platelet-derived growth factor receptor family

15. PTK Group XV
    Fibroblast growth factor receptor family
16. PTK Group XVI
    Insulin receptor family
17. PTK Group XVII
    LTK/ALK family
18. PTK Group XVIII
    Ros/Sevenless family
19. PTK Group XIX
    Trk/Ror family
20. PTK Group XX
    DDR/TKT family
21. PTK Group XXI
    Hepatocyte growth factor receptor family
22. PTK Group XXII
    Nematode Kin15/16 family
23. PTK Other membrane spanning kinases
    Other PTK kinase families

5. **OPK Group** - Other protein kinases (not falling in major groups)

    1. OPK Group I
       Polo family
    2. OPK Group II
       MEK/STE7 family
    3. OPK Group III
       PAK/STE20 family
    4. OPK Group IV
       MEKK/STE11 family
    5. OPK Group V
       NimA family
    6. OPK Group VI
       wee1/mik1 family
    7. OPK Group VII
       Kinases involved in transcriptional control family
    8. OPK Group VIII
       Raf family
    9. OPK Group IX
       Activin/TGFb receptor family
    10. OPK Group X
        Flowering plant putative receptor kinases and close relatives family
    11. OPK Group XI
        PSK/PTK "mixed lineage" leucine zipper domain family
    12. OPK Group XII
        Casein kinase I family
    13. OPK Group XIII
        PKN prokaryotic protein kinase family
    14. OPK Other
        Other protein kinase families (each with no close relatives)

1.2.3 Protein Kinase C superfamily

The family of protein kinase C enzymes includes 11 isozymes of Ser/Thr-specific protein kinases, which were first identified by the requirement of cofactors of diacylglycerol, Ca^{2+} and phospholipids for activation. The grouping of PKC isotypes based on both their structures and requirement of cofactors is useful for comparing the association of these enzymes at the protein level. PKC isotypes are categorized into five subgroups based on sequence alignment (Fig. 1.1) and this grouping is similar to the enzymatic grouping, with the only difference being the bisection of the nPKCs into two pairs of proximately associated kinases, namely, δ and θ, ε and η.

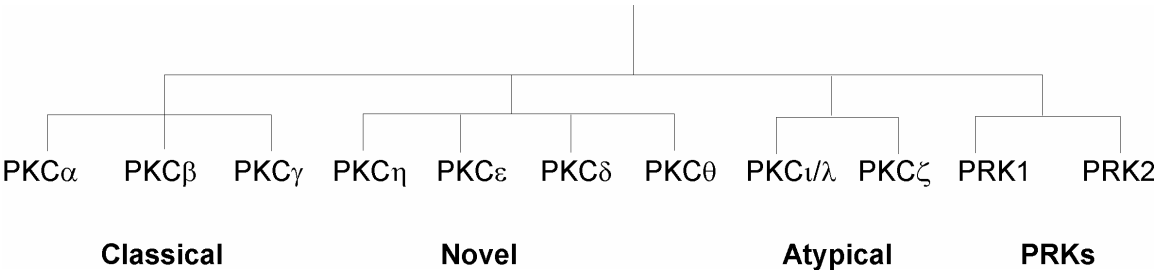


Fig. 1.1. Dendogram of the PKC superfamily

The first PKCs to be identified and cloned were the α, *β* and γ isotypes, which were initially isolated from rat brain cDNA libraries [34, 35]. Three additional PKCs, the δ, ε and ζ isotype were found via low-stringency screening of brain cDNA libraries with probes derived from the α, *β* and γ isotypes [36]. Subsequently, screens of other tissue cDNA libraries isolated PKCη [37], PKCθ [38] and PKCι (of which PKCλ is the mouse homolog). The PKC-related kinases (PRKs) cDNA was first identified as protease-activated serine/threonine kinases via conventional protein chemistry and were originally named protease-activated kinases (PAK). Later, PRK1 was isolated from rat liver and was shown to be able to phosphorylate S6 peptide and its analogue [39-41]. Subsequently, PRK1 and PRK2 were isolated from a human hippocampal

cDNA library by a low stringency plaque hybridization using the cDNA for the catalytic domain of PKCβII as a probe [42], or by PCR [43], and PRK protein was purified to homogeneity from rat testes using a specific antibody raised against recombinant PRK [44].

The mammalian PKC isotypes have been categorized into subclasses on the basis of the domain composition of the regulatory moiety, which in turn specify the cofactor dependence of the isozymes (Fig. 1.2).

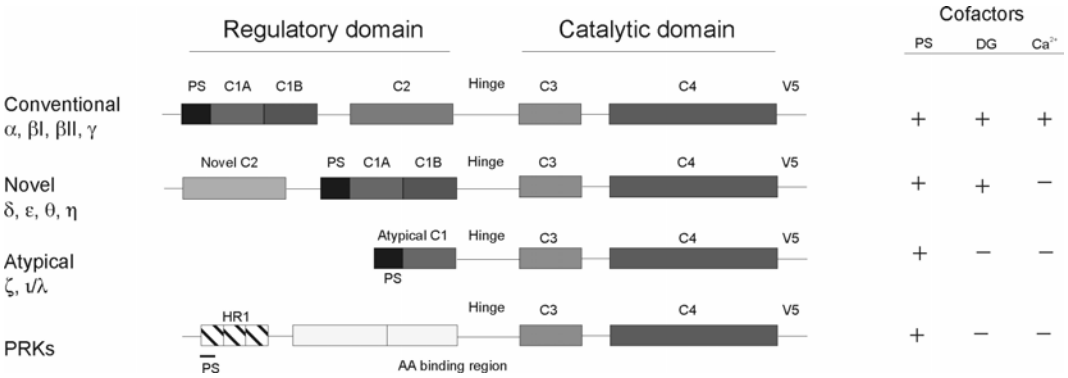


Fig. 1.2. Domain structures of the PKC subfamilies. PKC isotypes are made up of a regulatory and a catalytic domain, separated by a hinge region. The regulatory domain consists of conserved regions of C1 domain which binds phosphatidylserine and C2 domain which binds Ca^{2+}, while the catalytic domain is made up of the C3 which binds ATP and C4 domain which contains the substrate binding site. The C-terminal of all PKCs has a variable region (V5) which is highly variable in sequence and length. The requirements for cofactors for the different subclasses are shown on the right. PS, pseudosubstrate. AA, arachidonic acid. (adapted from [45]).

The most studied and understood of these groups is the conventional PKCs (cPKCs), which comprise the α, *β*I, *β*II and γ isotypes (the PKC*β* gene is alternatively spliced to generate two distinct proteins which vary only in their C-terminal [46]). These cPKC isotypes are activated by phosphatidylserine (PS) in a Ca^{2+}-dependent fashion; they also interact with diacylglycerol, which both increase the specificity of the enzyme for phosphatidylserine and also lowers the affinity for Ca^{2+} into the physiological range [47]. The cPKCs are activated by the tumour-promoting phorbol ester PMA, which

stimulates these enzymes by removing the need for diacylglycerol and lowering the threshold of Ca^{2+} needed for activation [48].

The novel PKCs (nPKCs) consist of the ε, η, δ and θ isotypes. These kinases are Ca^{2+}-insensitive, but are still regulated by diacylglycerol and/or phorbol esters in the presence of phoshatidylserine. The atypical PKCs (aPKCs), ι and ζ, constitute a third category. Similar to the novel PKCs, these protein kinases are Ca^{2+}-insensitive, nor do they respond to PMA/DAG. Instead they are activated by lipids, such as phosphatidylinositols and phosphatidylserine, and/or via interaction with Cdc42-GTP-Par6 complex [8, 49, 50].

Finally, the recently discovered PRKs constitute a fourth group with at least three members, PRKs 1-3. Resembling the aPKCs, all PRKs are insensitive to Ca^{2+}, DAG and phorbol esters [51, 52]. Instead, PRK 1,2 and 3 are activated by phospholipids and unsaturated fatty acids, notably by cardiolipin and arachidonic acid [44, 52-54]. At the same time, these kinases have been shown to be regulated by small G-proteins of the Rho class, through interactions at the N-terminal homologous region domains [55, 56]. It has been reported that Rho binding leads to increased activity (up to four fold) *in vitro* [57, 58].

1.2.3.1 Domain Structure

Each PKC isozyme consists of a single polypeptide chain having two structurally distinct domains: the amino-terminal regulatory moiety and the carboxyl-terminal catalytic core (app. 45 kDa). Each domain is further composed of specific modules. The regulatory moiety contains two key functions: an autoinhibitory sequence

(pseudosubstrate) and one or two membrane-targeting modules (C1 and C2 domains) that combine to regulate PKC activity and localization. The carboxyl-terminal region is the kinase domain and comprises two regions: the ATP binding lobe (C3) and the substrate binding lobe (C4). Separating the regulatory and catalytic domain is a hinge region which is susceptible to proteolytic cleavage by cellular proteases. Interspersed between the highly conserved regions (C1-C4) are divergent sequences known as variable regions (regions V1 to V5).

1.2.3.2 Pseudosubstrate

All PKCs have an N-terminal pseudosubstrate domain which confers auto-inhibition in the native enzyme. This module closely resembles the optimal substrate recognition motif of the PKC except that the serine/threonine is replaced by an alanine. In an inactive kinase, the pseudosubstrate domain binds to the substrate-binding pocket in the catalytic domain, thus achieving auto-inhibition [59]. Peptides based upon this sequence are competitive inhibitors of PKC, while peptide with a serine at the putative phosphor-acceptor position served as good substrates. Antibody developed against the pseudosubstrate sequence can activate PKC without cofactors, probably by retracting the pseudosubstrate from the active site.

In vivo studies provided evidence that indeed, regardless of PKC mode of activation, either via binding to its cofactors or by atypical processes such as binding cofactor-independent substrates, removal of the pseudosubstrate is a pre-requisite [60]. Further evidence comes from studies using proteases as conformational probes. Explicitly; the pseudosubstrate of PKC is resistant to cleavage in an inactivated kinase but when the kinase is activated, specific arginine residue in the pseudosubstrate sequence become

liable to cleavage [61]. Cleavage at the hinge removes the steric hindrance established by the autoinhibitory region such that, freed from the restraint; the catalytic domain becomes constitutively active. Energetics studies demonstrate that the energy required to liberate the pseudosubstrate region from the catalytic domain may be from the engagement of the membrane-targeting modules onto the membrane [62]. Thus to activate the kinase, the pseudosubstrate must be removed.

1.2.3.3 Membrane targeting modules

PKCs epitomize the use of two membrane-targeting modules (C1 and C2) to provide sensitive, specific, and reversible regulation of protein function. PKCs have provided insights that the collaborative engagement of both the membrane-targeting modules is a fundamental mechanism to reversibly regulate cellular location. The role of these modules is not limited to just regulating the membrane binding and removal of the inhibitory region from the kinase domain, thus mediating substrate specificity and biological function of the kinase [63, 64].

1.2.3.3.1 C1 domain

All PKC isozymes contain one (C1) or two (C1A and C1B) globular C1 domains, which are Cys-rich regions of approximately 50 residues. The module has two structural variants: the typical C1 domain which binds phorbol esters/diacylglycerol found in conventional and novel PKCs and the atypical C1 domain, which does not bind these ligands, in atypical PKCs [65].

Structural data from crystal [66] and NMR [67] analysis suggest that the binding site of the C1B domain is formed with two separated beta pockets (Fig. 1.3) to form a

globular domain. Conventional PKCs co-ordinate four Zn^{2+} atoms, two per C1 domain. The two ion binding sites play a key role in maintaining the folds of the C1 domain by bringing together residues that are far removed in the primary sequence. One metal binding site is near the bottom of the globular domain consisting of residues at the N and C termini of the domain, and the second site is in the middle of the molecule. In atypical C1 domains, the consensus sequences present in typical C1 domain are not present, thus compromising the structure in the binding site and the binding of phorbol esters or diacylglycerol [68]

Through a combination of structural analysis and site-directed mutagenesis, the molecular basis of phorbol ester binding has been established [66, 67, 69]. Ligand binding does not induce significant changes in the conformation of the cysteine-rich domain, but rather 'caps' a hydrophilic site at the top of the structure, forming a contiguous hydrophobic region that promotes insertion of the domain into the lipid bilayer. Specifically, in the absence of phorbol binding, the top half of the C1 domain is relatively hydrophilic because of the water-lined groove formed by the separated beta sheets. Once PMA binds to this groove, the domain is "capped" so that the top third provides a contiguous hydrophobic exterior. Thus, membrane targeting is elegantly achieved by ligands changing the surface properties of the module.

1.2.3.3.2 C2 domain

Although first described for the cPKCs, the C2 domain had been recognized as a widespread domain among numerous proteins (E.g. synaptotagmins, phospholipase C isozymes, phospholipase A_2, rabphilin-3A, Unc-13), with most of them related to signal transduction mechanisms or membrane trafficking [70]. As with the C1

domain, there is a second messenger-regulated and a second messenger-independent variant of the C2 domain. In conventional PKCs, this 12 kDa domain binds anionic phospholipids in Ca^{2+} dependent manner. Novel C2 domains lack key residues involved in Ca^{2+} binding and, as a consequence, novel C2 domains do not bind Ca^{2+} and phospholipids.

The C2 domain has two topological folds that differ only in that the first strand of one topology corresponds to the eighth strand of the second topology as a result of different positions for the amino and carboxyl termini: the type I topology exists for domains that follow the C1 domain (i.e. conventional isozymes) and the type II topology exists for domains that precede the C1 domain (i.e. novel isozymes) [70]. Despite a marked variation in primary sequence, structural analysis has revealed that all C2 domains fold similarly into a structure consisting of two four-stranded anti-parallel β-sheets connected by variable loops at the end of each strand to form a pocket, with the Ca^{2+} binding site located at one end of the domain [71, 72]. In Ca^{2+} responsive C2 domains, this pocket is lined by multiple aspartic acid residues that coordinate 2-3 Ca^{2+} ions. In novel C2 domains, key aspartic acid residues are missing and the domain does not bind Ca^{2+} [73].

The C2 domain binds membranes via residues that are on, or near, the loops that comprise the Ca^{2+} site [72]. Whether Ca^{2+} serves as a bridge in the lipid interaction is unknown. However, it is worthwhile to note that novel PKCs do not require Ca^{2+} for membrane binding and, since their C2 domain is missing critical aspartates in the Ca^{2+} binding groove of the C2 domain, the lipid interaction is not mediated by a Ca^{2+} bridge for these PKCs [73].

A number of functions in addition to membrane interaction have been ascribed to C2 domains, with many of the determinants mediating these other functions localized to the basic sheet behind the Ca^{2+} site. For example, peptides based on sequences in the basic sheet of PKC βII's C2 domain compete with binding to the PKC anchoring protein, RACK 1 [74]. Consistent with this, cloned putative IP4 receptor contains two C2 domains [75], and peptides derived from the basic face of the C2B domain effectively bind IP4 [76]. Recently, a study provided evidence that the C2 domain of PKCδ directly binds to phosphotyrosine peptides in a sequence specific manner, thus directly linking tyrosine phosphorylation with serine/threonine phosphorylation [77]. Thus the C2 domain may form a multi-functional module, with membrane targeting functions localized to the Ca^{2+} binding loops, and additional functions arising from the basic face or other regions of the domain.

1.2.3.4 HR1 domain

The homologous region 1 (HR1) domain was first identified in the regulatory region of PRK1, based upon sequence conservation with PRK2. This domain is composed of three repeats of an approximately 55-amino acid motif [43]. The first of these repeats, HR1a, is now known to incorporate the inhibitory pseudo-substrate site [78].

Protein alignments between PRK1 and PRK2 show that each motif seems to consist of two blocks of sequence with a short intervening linking sequence (Fig. 1.3). The first repeat is made up of basic amino acids which have a predicted α-helical structure. A short, partially conserved sequence of Gly-Ala-Glu-Asn acts as an intervening sequence. The second portion of the motif shows high homology to a leucine zipper in

terms of primary sequence. However, this does not lead to the folding of leucine zipper *in vivo* due to presence of helix breaker residues of glycine and proline.

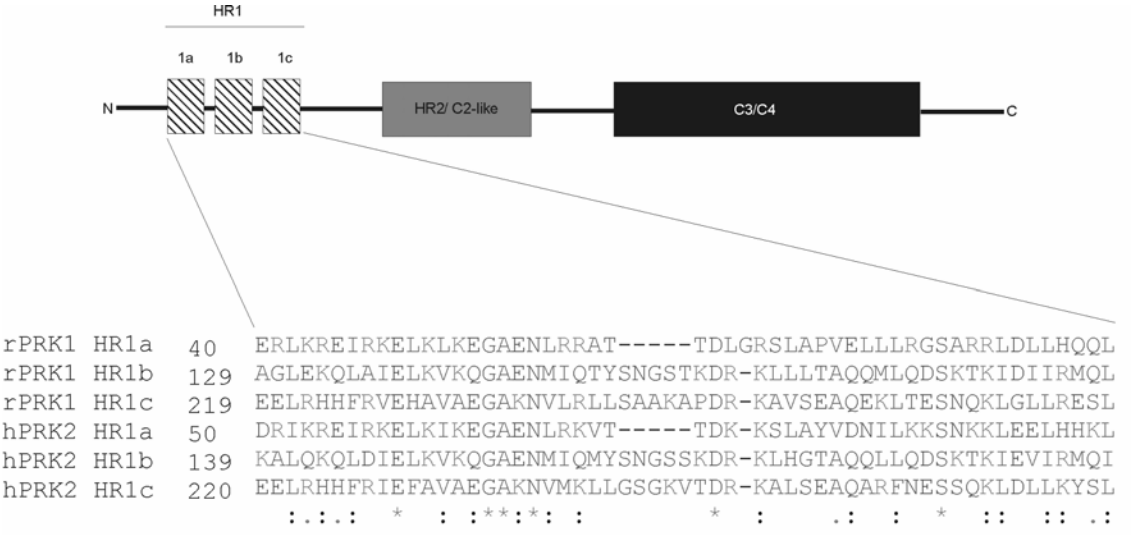


Fig. 1.3. Sequence conservation in HR1 motif. The HR1 domain is made up of three repeats: 1a, 1b and 1c. Sequence alignment among these three repeats in both PRK1 and PRK2 shows conservation of key residues. "*" means that the key residues in the column are identical in all sequences in the alignment. "." means that semi-conserved substitutions. ":" means conserved substitutions.

The involvement of small G proteins as binding partners for PRK1/2 was first defined when the HR1 motif was found to be a recurring sequence in two other RhoA-binding proteins, rhophilin [58] and rhotekin [79]. The minimal region required for Rho binding was found to be residues 33-111 in PRK1, which contain the first repeat, HR1a and the pseudo-substrate site [55]. Rho was subsequently found to bind to both isolated HR1a and HR1b domains but to HR1a in a more GTP-dependent manner, whereas no interaction was detected for HR1c [56]. G protein-interacting proteins are classically defined as effectors if they bind selectively to the GTP-bound (active) form of the G protein. These suggest that the HR1a repeat is the important Rho-binding site as it provided discriminated binding in favour of GTP-bound RhoA. The HR1c repeat does not bind Rho [56], and currently, its function is unknown. A possible role for HR1c may be to provide tighter binding to Rho through the combined contacts of

HR1a, b and c, since small protein motifs are often stacked together to form more stable domains [80].

Both PRK1 and -2 have been demonstrated to be targets not only for Rho but also for Rac [81, 82] and RhoB [83]. While PRK1 is targeted to the endosomal compartment by RhoA, localization studies have shown that the wild-type RhoB (of which only a fraction is in the GTP-bound state) is as efficient at targeting PRK1 to endosomes as a constitutively GTP-bound mutant RhoB [83]. This suggests that a weak interaction between PRK1 HR1b and RhoB is sufficient to confer binding *in vivo* and that the HR1 domain can act as localization signal as well as function in controlling kinase activation.

In this respect it is important to note that the published binding studies have been performed with recombinant RhoA which lacks the C-terminal prenylation modification found in the authentic molecule. It is possible that further contact points are made with the modified Rho that may require the complete HR1abc domain (or indeed other PKC domain). Alternatively, the HR1c motif may confer binding to some other proteins, perhaps another member of the Rho GTPase family.

1.2.3.5 Catalytic Domain

1.2.3.5.1 Kinase Core

The kinase domains of PKC have significant similarity to that of the archetypal kinase, PKA and PKB/Akt, with slightly more than 40% overall sequence identify. The architecture of the kinase domain was elucidated with the crystallization of PKA in 1991 [84] and PKB/Akt in 2002 [85]. There is a common structural fold of the kinase domain which consists of a bilobal structure with an N-terminal lobe that is

primarily composed of β sheet, and a C-terminal lobe that is primarily α helix [28, 86]. The ATP- and substrate-binding site is tucked in a groove between the two lobes. However, the kinase domain of PKC remained refractory to crystallization until recently when the structures of the catalytic domain of a member of the novel PKC subfamily, PKCθ [87] and a member of the atypical PKC subfamily, PKCι were solved [88]. The overall tertiary structure of the kinase domain of both PKCθ and PKCι displays the conserved fold of all other protein kinase structures solved so far: a bilobal kinase fold, with a small N-lobe, a large C-lobe and a hinge-linker.

The N-lobe of PKCθ is based on a five-stranded β-sheet (β1-β5) and two α-helices (αB and αC), and the C-lobe is mostly helical consisting of eight α-helices (αD-αK). The ATP-binding site, with the adjacent peptide-substrate binding site open to solvent, constitute the active site cleft at the interface of the two lobes. Although there is no observable electron density for regions corresponding to the turn motif and C-terminal to hydrophobic motif, superposition of PKCθ structure with PKA reveal similarity in overall structure.

For PKCι, the N-lobe consists of a five stranded anti-parallel β-sheet, two α-helices (αB and αC) and two short 3_{10}-helices. The C-lobe is mainly α-helical, with eight α-helices (αD-αJ, α-helix in turn motif) and three 3_{10}-helices. The C-terminal tail consisting of 72 residues (516-587) is wrapped around the surface of both lobes (Fig. 1.4). The first 14 residues (residues 517-530), are firmly attached to the C-lobe, while the latter residues (552-587) interact with the N-lobe. As with PKCθ, the aligned structures of PKCι with PKA display good agreement in overall conformation.

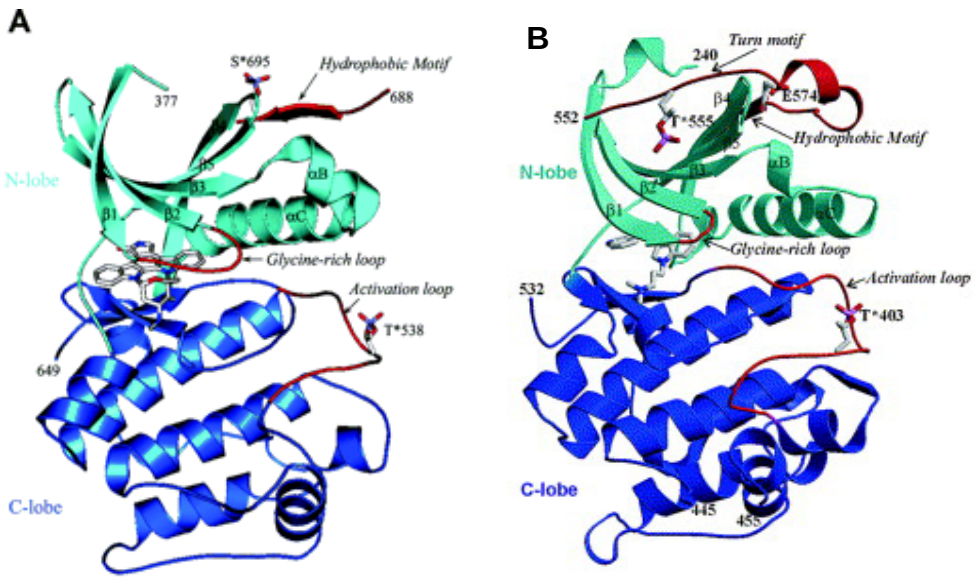


Fig. 1.4. Ribbon plot of the catalytic domain structure of PKCθ (A) and PKCι (B). The N-lobe is cyan and the C-lobe is blue. The activation loop, turn motif and the hydrophobic motif segment are displayed in red (from [88] and [87]).

1.2.4 Phosphorylation in the kinase core

Considerable information concerning phosphorylation sites on various kinases and their role in regulation of the enzyme now exists. The ABC kinases share three conserved phosphorylation motifs that must be processed sequentially for the enzyme to gain catalytic competence and correct intracellular localization [89, 90]. Thus, these sites act as phosphorylation-regulated switches to control intra and intermolecular interactions and to prime the kinase to be catalytically competent.

1.2.4.1 Activation loop site

Phosphorylation at a segment near the entrance to the active site provides an important regulatory mechanism throughout the protein kinase superfamily [28, 86]. The initial, rate-limiting step is the phosphorylation of the activation loop, on a threonine residue. Phosphorylation of this site is required for protein kinase activity.

Phosphate at this conserved segment not only precisely positions key arginine residues for catalysis, it also uncovers the access to the substrate binding site [84, 86, 91]. Kinetic studies showed a dramatic increase in phosphoryl transfer rate following activation loop phosphorylation [92]. Mutation of the critical threonine residue to a neutral, non-phosphorylatable residue inhibits the activity of the kinases, whereas mutation to an aspartate residue in the case of PKA and PKB/Akt, or to a glutamate residue in the case of PKC, results in the formation of a catalytically competent kinase [93-96]. In PKA, this site can be modified by autophosphorylation [93]. In the case of PKC, this reaction depends on an upstream kinase: PDK-1 [97-100], however, in PKCθ, there is evidence to suggest that the activation loop phosphorylation may be autophosphorylated [87].

1.2.4.2 Turn motif

Subsequent to the activation loop phosphorylation, phosphorylation of a motif in a Pro-rich domain follows. Unlike the activation loop, this site is modified by autophosphorylation [101, 102] and it corresponds to Ser338 in PKA which is positioned at the apex of a turn on the upper lobe of the kinase domain. This phosphorylated residue anchors the C-terminal tail to the top of the kinase lobe, with the phosphorylated residue located at the crest of a sharp turn, hence the name 'turn motif'. The crystal structures of PKCθ and PKCι provided evidence that this site occupies a similar position in PKCs. The structural studies imply that the phosphorylation at the turn motif is critical in stabilizing the kinase domain by fixing the C terminus at the top of the upper lobe of the kinase core [87, 88]. Negative charge at the turn motif is essential and adequate for kinase activity. Kinases with dephosphorylated activation loop and hydrophobic motif are still catalytically

competent [89]. However, mutation of the turn motif to alanine does not completely abolish activity due to other adjacent serine/threonine sites that can undergo compensating phosphorylation [103, 104]. In addition, the sequence encompassing this motif corresponds to a 14-3-3 binding site, thus suggesting a possible protein-protein interaction site [105].

1.2.4.3 Hydrophobic motif

The third phosphorylation site contains a serine or threonine residue flanked by hydrophobic residues, which is autophosphorylated following phosphorylation of the turn motif. This hydrophobic phosphoryation site is the least conserved of the three processing sites, with atypical PKCs having a glutamic acid (as phosphate mimic), at the phosphor-acceptor position. The sequence of PKA ends at the hydrophobic residue (phenylalanine) immediately preceding the phosphor-acceptor site. The crystal structure of PKA reveals that this terminal phenylalanine residue is tucked into a hydrophobic pocket on the back of the active site [106]; thus the back side of the kinase core has a binding pocket for the hydrophobic core. Structural studies of PKCθ and PKCι suggest a model in which the negative charge at the phosphor-acceptor site of the hydrophobic motif triggers an intramolecular 'clamp' with the upper lobe of the kinase domain. This not only serves to align residues more favorably, but also allows stabilizing contact with the activation loop [85, 87, 88, 107], which together promote catalysis.

In addition to stabilizing the kinase for activity, the hydrophobic motif is proposed to provide a docking site for PDK-1. Studies by Alessi [108] provided evidence that PDK-1 interacts with high affinity with sequences corresponding to the hydrophobic

phosphorylation motif, by identifying the C-terminus of PRK2 in a yeast two-hybrid screen for PDK-1 binding partners. Subsequently, it has been shown that the hydrophobic motif is the docking site of PDK-1 for a variety of substrates, including PKC and p70S6 kinase [109, 110]. In a study of PKCβII, it was suggested that in the unphosphorylated form, the hydrophobic site is exposed for PDK-1 binding but once phosphorylated, this site is hidden in an inactive kinase conformation [110].

1.2.4.4 V5 domain

The V5 domain, despite containing several highly conserved residues, has the lowest overall amino acid sequence identity among PKC. PKC are part of the AGC family of serine/threonine protein kinases [86]. Biochemically and structurally, PKA is perhaps the best studied protein kinase [111]. As Fig. 1.5 shows, the sequence of PKA terminates at Phe^{350}, which corresponds to the invariable phenylalanine residue NH_2-terminal to the phosphor-acceptor residue at the hydrophobic motif found in many AGC kinases [112].

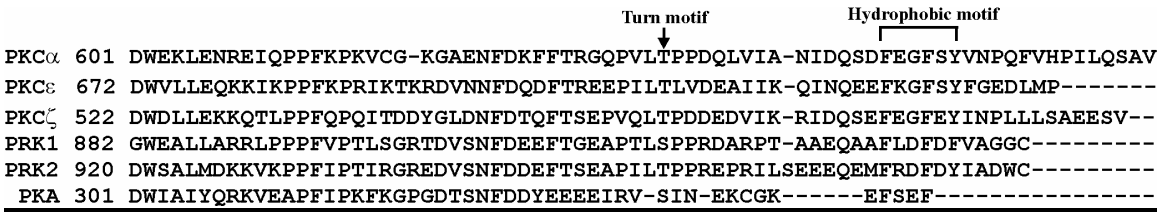


Fig. 1.5. Alignment of amino acid sequences of V5 domains of representative isozymes in the PKC superfamily with that of PKA. V5 domains from representative isozymes from cPKC (bovine PKCα) [35], nPKC (mouse PKCε [113], aPKC (rat PKCζ) [49] and PRKs (rat PRK1 [114]) and human PRK2 [43]) were aligned with that of human PKA [115]. Amino acid residue numbers are shown to the left of the sequences.

Although too short (approximately 50 amino acids) to be considered a protein domain, the V5 domain of PKC and PRK plays an important regulatory role in kinase function. The initial identification of the V5 region was based upon the decrease in

sequence homology among PKC α, β and γ at the extreme C-terminus of the kinase domain [34]. Primarily, interest in the V5 region stems from PKC βI and II, which are differential spliced products of the same gene and differ only in the V5 region [116, 117]. PKC *β*I and *β*II are shown to be localized differentially both in their active and inactive state [116, 117] and translocate to different intracellular compartments following PMA treatment [118]. The isolated V5 domain of PKC βI suppresses proliferation of neuroblastoma cells more than that from PKC βII [119]. Chimeras generated between various regions of PKC α and βII demonstrated that the COOH-terminal 13 amino acids from PKC βII are sufficient to confer the nuclear translocation and lamin B phosphorylation characteristic of PKC βII [120, 121]. Further studies showed a selective interaction between this region and the nuclear membrane lipid, phosphatidylglycerol, which may account for the specific nuclear function of PKC βII [120]. In addition, the V5 region in PKC βII is shown to bind to the selective anchoring protein, RACK1, and the inhibition of this interaction by peptides derived from the βII region abolished PKC βII mediated cardiac myocyte hypertrophy [122].

The PDZ (PSD-95, Dlg and Zo-1) domain is found in a variety of scaffolding proteins that mediate the organization of signaling networks [123]. Thus far, three PKC binding proteins that contain PDZ domains have been identified, namely, PICK1, INAD and ASIP, which interact with the V5 domain of PKC isozymes. PICK1 was isolated in a yeast two-hybrid screening, using the catalytic domain of PKCα as bait [124]. PICK1 localizes PKCα to a perinuclear region [124, 125]. The *Drosophila* scaffolding protein INAD contains five PDZ domains, two of which interact directly with *Drosophila* eye PKC [126, 127]. The PKC binding site for INAD had been

mapped to the carboxyl-terminal region of PKC, although sites outside the V5 region may also be involved [127]. Finally, the third PDZ-containing protein ASIP, which was recently identified by Ohno and co-workers [128], is a novel atypical PKC-specific interacting protein. This protein contains three PDZ domains. ASIP localizes atypical PKCs to tight junctions and may play a role in the maintenance of epithelial cell polarity.

In addition, the V5 regions of PKCs are critical in phosphorylation. This region incorporates two key phosphorylation sites (the turn and the hydrophobic motif), both of which are critical in priming the kinase. Phosphorylation of these sites controls the accumulation of phosphate at other sites in PKCα, as well as contributing to the maintenance of the phosphatase-resistant conformation [129], which affect the rate of dephosphorylation in PKC α and β [103, 129, 130]. The basis for these effects seems to be in part due to the interaction of the phosphorylated V5 region with the kinase domain itself, inducing a closed, stable conformation.

The elucidation of the three dimensional structure of PKCθ and PKCι provides structural prototypes for the entire protein kinase family [87, 88]. Studies on the conformations of active PKCs show that the V5 domain of the kinases contributes to substrate recognition and substrate-enzyme complex stability by helping to secure the kinase core, especially the ATP-binding site, in a functional conformation. This is done by wrapping back the C-terminal tail around the lower and upper lobes of the kinase domain. Indirect evidence that this region of PKC α is important for stablility/activity derives from a deletion analysis of the mammalian protein expressed in *Saccharomyces cerevisiae*, where removal of just the C-terminal 26 amino acids

destroys activity [131]. It is noteworthy that the most C-terminal phosphorylation site in the V5 regions of PKCs (the so-called 'FSY' site) is replaced by a glutamic acid residue or an aspartic acid residue in the aPKCs and PRKs, respectively. In PKC α the equivalent mutation (S657D) partially retains wild-type PKC α properties [129]. Thus it appears that for aPKCs and PRKs, the requirement for a phosphate residue on this hydrophobic site is by-passed.

1.3 Activation

1.3.1 PKC activation *in vivo* by membrane translocation

PKC is readily recovered as a soluble protein from resting cells homogenized in the presence of EDTA [132]. Upon treatment of cells with agonists leading to the stimulation of phosphatidylinositol-specific phospholipase C activity, a redistribution of PKC from cytosol to the plasma membrane is observed. Treatment of parietal yolk sac cells with phorbol ester elicits a rapid decrease in cytosolic PKC that is accompanied by a significant increase in the level of plasma membrane-associated enzyme [133]. A rise in either internal Ca^{2+} or DAG can cause cytosol-membrane translocation of PKC [134, 135]. These results established a temporal correlation between the intracellular distribution of PKC and its putative activation state. Phorbol esters are potent activators of PKCs. They can mimic the effects of the natural activator of PKCs, diacylglycerol [136]. It is widely recognized that phorbol esters are promoters of skin tumors, with pleiotropic and often tissue specific cellular responses such as proliferation [137], differentiation [138], apoptosis [139] and gene expression [140]. PKC has been identified as the high-affinity intracellular receptor of phorbol esters. Phorbol ester activates PKC *in vitro* and can substitute for DAG in platelet activation [141]. DAG can inhibit phorbol ester binding to PKC competitively,

indicating that the sites of interaction for these activators are similar if not identical [142]. Since phorbol esters have more than 1000-fold higher affinity than DAG for PKC in the presence of lipid [143], they have been used extensively in many PKC assays.

1.3.2 Lipid-induced PKC activation

Pseudosubstrate sequences within the intramolecular regulatory domain have been identified in PKCs. These sequences provide a means of autoinhibitory activity of proteins by interacting with the catalytic domain [144, 145]. The interaction between specific lipids or other activators and the regulatory domain of the PKCs leads to conformational changes that release autoinhibitory restraints imposed by the pseudosubstrate domain and induce PKC activation [60]. Some lipid activators of PKC are illustrated in the following segments, including diacylglycerol, phosphatidyl-L-serine, and fatty acids.

1.3.2.1 Diacylglycerol (DAG)

cPKCs and nPKCs are activated by DAG and phorbol esters in the presence of phosphatidylserine (PS) [49, 146]. Activation of PKC by DAG analogues is very stereospecific: the *sn*-1,2-DAGs, but not the 1,3- or 2,3-DAGs are effective in assays using either lipid vesicles or mixed micelles [147, 148]. Both the oxygen esters (1, 2-position) and the primary hydroxyl group (3-position) are essential for DAG function. The acyl chain composition does not appear to be critical although a preference for unsaturated side chains has been reported [149-151]. The major requirement with respect to acyl chains appears to be their length. *In vitro* studies showed that DAG with both acyl chains containing six or more carbon atoms induces maximal kinase

activity, indicating that DAGs must be sufficiently hydrophobic to associate with membranes or micelles [152-154]. Activation of PKC by DAGs is believed to result from two effects. Firstly, the affinity of PKC for PS increases in the presence of DAG and strengthens PKC association with the membrane [155, 156]. Secondly, DAG together with PS induces the conformational changes that expose the pseudosubstrate domain [61].

1.3.3.2 Phosphatidyl-L-Serine (PS)

It has been almost 20 years since Nishizuka and coworkers reported that the pro-enzyme of the protease-activated kinase (PKC) is activated by PS [157]. PS is the phospholipid which is best able to activate PKC [158, 159] and is thought to represent the principal physiological phospholipid cofactor. The high affinity of PKC for PS in mixed micelles requires DAG. DAG can increase the affinity of PKC for PS [155]. Similarly, Ca^{2+} increases the affinity of PKC for PS [155]. The activation response curve of PKC by PS is sigmoidal. PS concentrations of 15 mol % of total lipid in the plasma membrane are sufficient to totally activate PKC in the presence of saturating amounts of Ca^{2+} and DAG. PKC interacts with multiple phosphatidylserine molecules. The actual number of PS molecules that interact with one PKC molecule has recently been calculated to be on the order of eight. Thus, PKC interaction with PS can cause extensive segregation of acidic phospholipids [160].

1.3.3.3 Other phospholipids

Several other negatively charged phospholipids such as phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylglycerol (PG) and cardiolipin (CL) are capable of supporting PKC activity although they are less effective than PS [154, 161]. In

contrast to the highly specific interaction between PKC and PS in the presence of DAG, the interaction between PKC and other phospholipid effectors appears to be due to less specific electrostatic effects, reflecting the capacity of acidic lipids in general to bind to PKCs [162]. Various PKC isozymes have distinguishable responses to these phospholipids in some instances. For example, cardiolipin is a strong activator of PKCγ and PKCε [163, 164]. The precursors of DAG, phosphatidylinositol-4,5-biphosphate (PIP_2), phosphatidylinositol-3,4,5-triphosphate (PIP_3) and phosphatidylinositol-3, 4-biphosphate have been reported to be potent and selective activators of PKCδ, ε, η [165, 166]. PIP_3 was also implicated in the activation of PKCζ [167]. Recent evidence demonstrates that PKCζ can be activated by ceramide, which is produced by stimulation of sphingomyelin hydrolysis catalyzed by sphingomyelinase, both *in vitro* and *in vivo* [168]. These data suggest that individual PKC isozymes can be regulated by more than one lipid and they may have separate and unique functions in the cell.

1.3.3.4 Fatty acids

In the absence of PS and Ca^{2+}, *cis*-unsaturated fatty acids such as arachidonic, linoleic, linolenic and oleic acid can activate PKC to varying degrees depending on the isozyme [159, 169-171]. Furthermore, fatty acids have been shown to activate PKC synergistically with DAG in the presence of PS and to produce nearly fully active PKC at basal cytosolic Ca^{2+} concentrations [172-175]. PKC activation by fatty acids is thought to be physiologically relevant but the mechanism of activation is distinct from that of DAG. Fatty acids do not interact with PKC at the phorbol ester/DAG-binding site [172, 173]. Fatty acids are usually produced by phospholipase A_2, while DAG is produced by phospholipase C-catalyzed hydrolysis of

phosphatidylinositol and phosphatidylcholine or via prior formation of phosphatidic acid through phospholipase D-catalyzed hydrolysis [176, 177]. Three major groups of phospholipase C, PLCβ, PLCγ, and PLCδ, have been identified in mammalian tissues, and each group consists of more than one isoform. The enzymes are activated upon cell stimulation and cleave inositol phospholipids to produce inositol phosphates and DAG needed for PKC activation. At a later phase of the cellular response, phospholipase D plays a major role in the production of DAG from PC to prolong PKC activation [178]. Lysophosphatidylcholine (lysoPC) is the product of PC hydrolysis by PLA_2. LysoPC together with DAG can significantly strengthen subsequent cellular responses [8]. DAG produced from PI hydrolysis is rapidly converted to phosphatidic acid (PA) by the action of DAG kinase, and further to PI, PIP, and PIP_2. PA produced from PC is converted to DAG by the action of phosphomonoesterase [177, 179, 180]. On the other hand, the DAG produced from PC hydrolysis is a poor substrate for DAG kinase, and is slowly degraded by DAG lipase [181].

In summary, PKC activation is a convergent point for various signal-induced pathways, such as membrane phospholipid hydrolysis products formed by the activation of phospholipases A_2, C and D [182]. These phospholipases may be activated by different stimuli at different locations in cells. In specialized cases, activation of PKC may also be regulated by sphingolipid metabolites [8, 183] or even ultraviolet B (UVB) [184, 185]. A recent study by Shen and colleagues demonstrated that although both pro-apoptotic PKC (PKCδ) and pro-survival PKC (PKCζ) were activated by UVB, specific and opposing regulation on both isoforms was achieved with a sesquiterpene lactone [185].

1.4 Post-translational processing and maturation

All known PKC isozymes can autophosphorylate [37, 38, 102, 146, 186-190]. Detailed studies of the role of autophosphorylation have mostly been carried out with cPKC isozymes and have revealed that the autophosphorylation is an intramolecular reaction at both serine and threonine residues on both regulatory and catalytic domains [191-193]. Autophosphorylation of PKC has a K_m value for ATP about 10-fold lower than that for substrate phosphorylation [191], similar to that observed for phosphorylation of exogenous substrates [194, 195]. At the same time, there appears to be a dependence on PS and DAG [4, 196]. The final step for PKC maturation is the autophosphorylation of serine/threonine residues in the turn motif and hydrophobic motif. After this, the PKCs become catalytically competent upon activation by cofactors such as DAG [45]. The competence of members of the PKC family to respond to lipid second messengers depends on a series of ordered phosphorylation events [197, 198]. The kinase domain of PKCs contains three conserved phosphorylation sites: the activation loop (XTXTFCG) which is phosphorylated by phosphoinositide-dependent kinase (PDK1) [97, 100], and two carboxyl-terminal sites, the turn motif and hydrophobic FXXFS/TF/Y motif which are regulated by autophosphorylation [101]. Phosphate at the activation loop unmasks the substrate binding cavity and structures the enzyme for catalysis, with phosphates at the two carboxyl-terminal positions then locking the enzyme in this catalytically competent conformation. Newly synthesized PKCs are in a conformation that promotes binding of PDK-1 to its carboxyl terminus and in which the activation loop is exposed for phosphorylation. Thus, the primary regulation of this phosphorylation event is the direct interaction of PKC with PDK1. Although phosphorylation at the activation loop is required for the maturation of PKC, once the enzyme is phosphorylated at the C-

terminal sites, the activation loop phosphate is dispensable [89]. The fully phosphorylated species of PKC is the mature form that is competent to respond to lipid second messengers.

It has been reported that protamine can stimulate autophosphorylation of PKC [199, 200], which is consistent with the role of protamine as an unconventional activator of PKC [201]. Protamine is thought to induce a conformational change that releases the pseudosubstrate domain from the active site, thereby leading to PKC activation [60]. Protamine sulfate induced phosphorylation occurs in the absence of phosphatidylserine, diacylglycerol, and calcium [157]. Protamine sulfate alters the structure of PKC in the same way as lipid activators, thus effecting the same change in function: activation. Protamine sulfate can expose Arg^{19} of PKCβII's pseudosubstrate domain to proteolysis [199].

The active species of PKC is highly sensitive to dephosphorylation: biochemical studies have shown that membrane-bound PKC is two order of magnitude more sensitive to dephosphorylation than is soluble PKC [197]. In fact, prolonged activation by treatment of cells with phorbol esters results in dephosphorylation and down-regulation of PKC [202]. The dephosphorylated species localizes to the detergent-insoluble fraction of cells, where it eventually proteolysed. Studies with PKCα suggest the involvement of caveolin-dependent targeting to endosomes [203]. Limited evidence suggests that proteolysis may be through an ubiquitin-mediated pathway [204, 205]. Treatment with phorbol esters is frequently used to 'down-regulate' PKC in experiments aimed at addressing the involvement of PKC in cell

signaling [206]. Atypical PKCs are resistant to this phorbol-ester mediated down-regulation.

Although the phosphorylation of conventional PKCs is constitutive, their dephosphorylation may be under agonist control [202]. Both phorbol esters and ligands such as tumour necrosis factor α (TNFα) result in PKC inactivation and dephosphorylation [207-209]. Furthermore, serum selectively promotes the dephosphorylation of the activation loop segment in conventional PKCs, thus uncoupling the phosphorylation of the activation loop from that of the C-terminal sites [210]. The hydrophobic site of PKCε has also been reported to be selectively dephosphorylated by a rapamycin-sensitive phosphatase [211]. Recent studies have shown that the dephosphorylated turn motif provides a specific binding site for the molecular chaperone, Hsp70 [212]. This dephosphorylated-dependent binding of Hsp70 stabilizes PKC and allows it to become rephosphorylated and cycle back into the pool of functional PKC. Disruption of the interaction of Hsp70 with PKC results in accumulation of dephosphorylated PKC in the detergent-insoluble fraction of cells, which are subsequently targeted for degradation.

1.5 Isozyme specific regulation

The existence of PKC heterogeneity poses the question as to why so many isotypes exist and what the functional relevance of each of them could be. The high degree of conservation of the individual PKC isotypes over various mammalian species indicates that maintaining heterogeneity is useful and suggests specific roles for each isotype. Currently, isozyme specific regulation is proposed to be mediated by substrate specificity, localization and cofactor dependence.

1.5.1 Substrate specificity

A large number of proteins have been shown to be phosphorylated either *in vitro* or *in vivo* by PKC [213]. Based on these data as well as studies using synthetic peptides, a basic consensus PKC phosphorylation motif was originally determined: RXXS/TXTX, where X indicates any amino acid. These studies underscored the importance of basic residues N- and C-terminal to the phosphor-acceptor residue. Similarly, a large number of 'artificial' substrates of PKC are also known and used for activity measurements, such as myelin basic protein, various histones, protamine and protamine sulfate. Recently, an oriented peptide library approach was used to determine the optimal substrate selectivity of nine PKC family members [214]. The results confirmed the requirement for basic residues surrounding the Thr or Ser, and indicated that although the optimal phosphorylation for each PKC is similar, there are some subtle differences between family members, particularly between conventional, novel and atypical PKCs. All PKCs prefer basic residues at positions -6, -4 and -2 to the Ser/Thr. cPKCs also prefer basic residues at +2, +3 and +4, whereas nPKC and aPKC prefer hydrophobic residues at these positions. The only deviation to this selectivity was PKCμ, which had a strikingly distinct optimal motif from all other PKCs, with a strong selectivity for Leu at the -5 position [214]. This approach has provided an explanation for the substrate selectivity of certain PKCs over other family members. For example, the MARKCS protein (myristoylated alanine-rich C-kinase substrate), a ubiquitous PKC substrate in all cells, is phosphorylated by all PKCs except PKCζ [215]. Similarly, plectstrin (platelet and leukocyte C-kinase substrate protein), a PKC substrate found in cells of hematopoietic origin, appears to be phosphorylated by all PKCs [216]. Conversely, Sheu and colleagues [217]

demonstrated that although all members of cPKCs efficiently phosphorylate GAP-43 (growth-associated protein of 43 kDa, also known as neuromodulin), neuromodulin was a better substrate for PKCα and PKCβ than PKCγ. The human telomerase-associated protein 1 (hTEP1) and the telomerase catalytic subunit (human telomerase reverse transcriptase hTERT) were found to be selectively phosphorylated by PKCα [218] while the heterogeneous ribonucleo-protein A1 (hnRNP A1) was efficiently phosphorylated by PKCζ but not other PKCs [219], whereas elongation factor eEF-1α was a good PKCδ substrate [220]. In addition, Sheu et al [221] demonstrated that oxidized neurogranin was a poorer substrate for PKC; both the reduced and oxidized forms had similar K_m values, but the V_{max} of the oxidized form was about one-fourth that of the reduced form. Thus oxidation/reduction mechanisms may function as an additional level of specificity.

Despite these subtle differences, it is clear that all PKCs generally prefer to phosphorylate similar sequences, suggesting that other mechanisms must exist to discriminate which particular PKC isoform participates in signaling mechanisms.

1.5.2 Specific cellular localization

Since the early 1990s it has become clear that in addition to binding to lipids, PKC can also interact with proteins via protein-protein interactions. These interactions play an important role in the localization and function of PKC isozymes. PKC binding proteins are defined as proteins (which may or may not be PKC substrates) that bind PKC directly via a nonsubstrate binding site. These proteins may be grouped together on the basis of the conformation of the PKC required for binding, cofactor requirement, and whether or not the proteins are PKC substrates. PKC in an active

conformation will bind to receptor for activated C-kinase (RACKs) [222, 223] or substrates that interact with C kinase (STICKs) [224, 225], whereas inactive PKCs will interact with A kinase anchoring protein (AKAPs) [226] and 14-3-3 [227, 228]. PKC binding proteins may or may not be PKC substrates (e.g STICKs and RACKs, respectively) and may require cofactors for binding to PKC (STICKs require phosphatidylserine [224]; GAP-43 requires Ca^{2+} [229]). Such proteins serve many functions, which include localizing inactive (AKAPs) or active (RACKs) PKC isozymes to specific intracellular sites or serving as substrates (STICKs), shuttling proteins (RACK1) [223], PKC activator (syndecan-4) [230], or PKC inhibitors (14-3-3, Nef) [231, 232].

1.5.2.1 RACKs

RACKs were originally identified by Mochly-Rosen and co-workers, who in the early 1990s proposed that the intracellular localization of PKC isozymes upon activation is mediated via interaction with isozyme-specific anchoring proteins [233, 234]. RACKs were first characterized as Triton-X 100 insoluble proteins that bind PKC isozymes only in the presence of PKC activators [233, 234]. The PKC-RACK interaction is mediated, at least in part, by the C2 region in cPKCs and the C2-like region (within the V1 domain) in nPKCs [222, 235-237]. Two RACKs have been identified to date by using overlay assays: RACK1, which specifically interacts with PKC βII [74, 223], and RACK2 (β'-COP), which specifically interacts with PKCε [222]. Both RACKs bind PKC in its active conformation. Despite the differences in sequence between RACK1 and RACK2, the two proteins share common features. Although RACK1 and RAKC2 are not PKC substrates, both increase PKC phosphorylation of substrates [222, 223]. RACKs contain 'WD-40 repeats', a motif known to mediate protein-

protein interaction. It was observed that RACK1 translocates to the same site as activated PKCβII and specifically associates with this isozyme upon activation. These findings suggest a potential role for RACK1 as a shuttling protein [237].

1.5.2.2 STICKs

STICKs are an important group of PKC binding proteins that were discovered by Jaken and co-workers [238]. STICKs require phosphatidylserine for interaction and are PKC substrates. Phosphorylation of STICKs regulates their association to PKC. The identified STICKs include MARCKs, MacMARCKs, α-adducin, β-adducin, γ-adducin and clone 72 (SseCKs) [224, 238-240]. STICKs are involved in a variety of functions. For example, adducins are cytoskeletal proteins involved in the interaction between actin and spectrin. Expression, localization, and phosphorylated states of α-adducin and γ-adducin have been correlated with renal tumor progression [238-240]. Clone 72, a major PKC binding protein in REF52 fibroblasts, is involved in cytoskeleton modeling and cell growth [241]. Another STICK is the serum deprivation response protein (Sdr) that binds and localizes PKCα within the microdomain of caveolae [224].

1.5.2.3 Scaffolding proteins

Scaffolding proteins cluster signaling proteins, thus allowing a tight control of cellular pathways as well as cross-talk between different cascades. Scaffolding proteins such as caveolin, AKAPs and 14-3-3 cluster PKC to specific intracellular sites. PKC scaffolding proteins that have been identified to date bind to PKC in its active conformation.

1.5.2.3.1 Caveolin

Caveolin targets a variety of signaling proteins such as the α-subunit of G-proteins, Src and EGF receptors to the caveolae [242-244]. PKCα localizes to the microdomain of the caveolae [224, 244]. PKCα, in addition to PKCζ also associates with caveolin, whereas PKCε was not found to associate with this protein [244]. Caveolin interaction with PKC results in the inhibition of PKC activity. Moreover, a short peptide derived from caveolin interacts directly with PKC and inhibits its kinase activity [244].

1.5.2.3.2 AKAPs

Two members of the AKAP family of proteins that target PKA to a specific intracellular site are also scaffolding proteins for PKC. Elegant work by Scott and co-workers has demonstrated that AKAP79 assembles PKC, phosphatase 2B, and PKC at postsynaptic dendritic fractions [226, 245, 246]. AKAP79 inhibits PKC activity [226]. Ca^{2+}- calmodulin as well as DAG release PKC from AKAP79 [246]. In HEK-293 cells, AKAP79 is phosphorylated after PMA stimulation [247]. Phosphorylation of AKAP79 by PKC regulates its subcellular compartmentalization [247]. Thus, there is a reciprocal regulation of AKAP79 and PKC with regard to subcellular localization. Another AKAP identified by Scott and co-workers , AKAP250 (Gravin), assembles both PKA and PKC to the filopodia in human erythroleukemia cells [248].

1.5.2.3.3 14-3-3

14-3-3 is a family of highly homologous proteins that are ubiquitously expressed. The role of 14-3-3 proteins in PKC signal transduction remains controversial. 14-3-3 isozymes may either inhibit [227, 249] or enhance PKC activity [228, 250]. The dimeric structure of 14-3-3 enables the protein to serve as an adaptor or scaffold for a variety of signaling proteins, including PKC [251]. Meller et al [231] have reported a specific interaction between a PKC isozyme (PKCθ), which may help keep this PKC isozyme in its inactive conformation. 14-3-3τ may represent an example of RICK (a receptor for inactive C kinase), which binds to PKC and targets the inactive isozyme to specific intracellular sites.

1.5.2.4 Direct interaction of PKC isozymes with cytoskeletal proteins

PKC isozymes associate with cytoskeletal proteins and this interaction between PKC and cytoskeletal proteins is at least in part isozyme-selective. An example of this isozyme specificity is PKCζ, which associates with tubulin via the pseudosubstrate region [252]. Terrain and co-workers have demonstrated that PKCε specifically binds F-actin via an actin binding site within the C1 region [253, 254]. F-actin activates PKCε in the absence of phospholipids [253]. PKC βII (but not PKC βI) also interacts with F-actin via its V5 domain and tranlocates to the actin cytoskeleton upon activation [255]. PKC βII selectively phosphorylates actin, although actin is a poor substrate. The interaction of PKC βII with actin results in a significant enhancement in autophosphorylation and in an alteration in substrate specificity [255]. Furthermore, the interaction between PKC βII and actin protects PKC from degradation and down-regulation [255]. The interaction between PKC and F-actin was also observed in *Aplysia* [256]

1.6 Protein Kinase C Related Kinase (PRK)

1.6.1 History and structure of PRK

Protein kinase C-related kinases (PRKs) were first identified as protease-activated serine/threonine kinases via conventional protein chemistry and were originally named protease-activated kinases (PAK). They were isolated from rat liver and were shown to be able to phosphorylate S6 peptide and its analogue [39-41]. Later, PRK cDNAs were isolated from a human hippocampal cDNA library by low stringency plaque hybridization using a cDNA fragment for the catalytic domain of PKCβII as a probe [42] or by PCR [43] ,or with specific oligonucleotide primers [114]. PRKs have 50 % sequence similarity in the catalytic domain to protein kinase C [43, 257]. PRK but not PKN is adopted as the preferred nomenclature because before Mukai and Ono used PKN to describe this kinase, another serine/threonine protein kinase which is a basic 45-47 kDa kinase activated by NGF and several other factors in PC12 cells and other cell types had already been named PKN [258]. Secondly, to reflect the homology to PKCs and to prevent confusion, PKC-Related Kinase (PRK) may be a more suitable nomenclature than PKN. The structures of the isoforms of PRK are shown schematically in Fig 1.6.

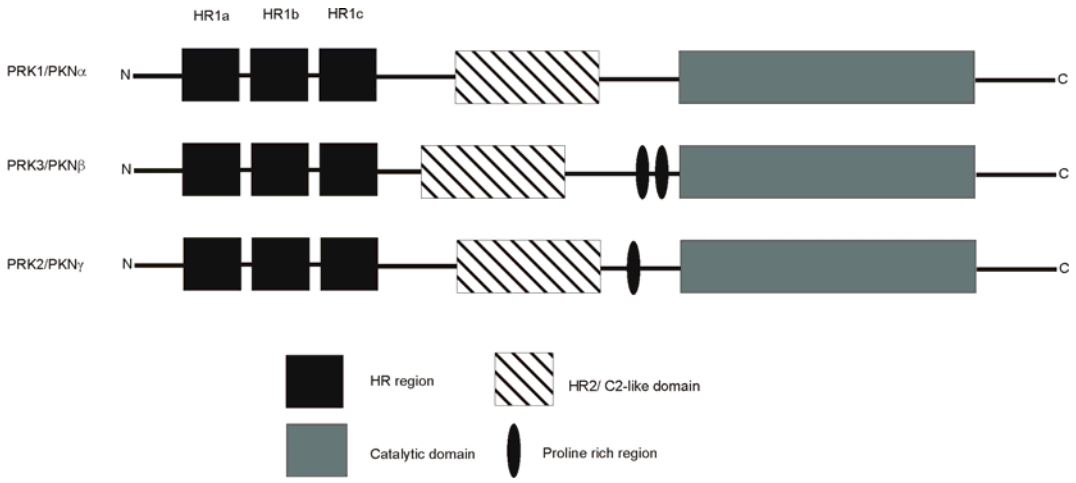


Fig 1.6. Primary structure of PRKs (adapted from Mukai [259]).

1.6.2 Distribution of PRK

PRK1 mRNA is expressed ubiquitously in human and rat tissue [42, 260]. Mouse PRK2 mRNA was detected ubiquitously in tissues by Northern blotting [261] and whole body situ hybridization while human PRK2 mRNA was reported to express specifically in a DX3 lymphoid lineage by Northern blotting and RT-PCR experiments [43]. In contrast, PRK3 mRNA was almost undectable in normal human tissues. Levels were especially abundant in chronic myelogenous leukemia K-562, colorectal adenocarcinoma WE480, and HeLa cells [262]. PRK1 level in the rat brain was found to be approximately 0.01% of total protein and biochemical fractionation revealed that the half of the total PRK1 is distributed in the soluble cytosolic fraction, and one-third of it is in a post-nuclear membrane fraction [44]. PRK1 was found to be especially abundant in the neurons of human brains, with a concentration in the endoplasmic reticulum (ER) and ER-derived vesicles localized to the apical compartment of the apical compartment of the juxtanuclear cytoplasm, late endosomes, multivesicular bodies, Golgi bodies, secretory vesicles and cell nuclei

[263]. On the other hand, PRK3 was detected in the cell nucleus and the perinuclear region, but not in the cytoplasmic region [262].

1.6.3 Regulation of activity

Activators of PKC such as calcium, phosphatidylserine/diolein do not enhance PRK activity [44, 54]. On the other hand, unsaturated fatty acids such as arachidonic and linoleic acids were identified as potential activators of PRK [52], albeit PRK2 is significantly less sensitive to arachidonic acid than PRK1 [262, 264]. Cardiolipins, phospholipids such as phosphatidylinositol 4,5-biphosphate (PI4,5-P2) and phosphatidylinositol 3,4,5-triphosphate (PI3,4,5P3), and lysophospholipids such as lysophosphatidic acid (LPA) and lysophosphatidylinositol can also stimulate the kinase activity of PRK1 and 2.

It has been reported that the GTPase RhoA bind to PRK1 in a GTP-dependent manner and the activity of purified bovine brain PRK1 is stimulated in the presence of a GTPγS bound form of RhoA [55-58]. PRK1 has been reported to interact with Rac but interaction is weak with negligible activation [56-58, 82]. As for PRK2 binding with Rho family GTPase, there exist different schools of thought, with one reporting that the binding is GTP-dependent [261] and the other reporting the binding to be nucleotide-independent [82].

The N-terminal region of PRK is thought to act as an inhibitory region to the kinase activity, of which the binding of activators causes the inhibition to be lifted [52, 78, 114, 265]. The prevailing model by RhoA activation proposes the binding of RhoA to the ACC finger domain removes the pseudosubstrate region from the catalytic domain

and thus presents an active kinase domain [266]. However, an N-terminal deleted mutant without the three anti-parallel coiled coil (ACC) fingers has a basal activity similar to wild type kinase [267]. The exact mechanism by which PRK is activated by RhoA to produce conformational changes is still unknown. Recently, Parekh et al. [198] proposed a model of Rho dependent PRK activation via PDK1 based on the data reported in [268]. Activated Rho binds to PRK, to disrupt the autoinhibitory effect mediated by the pseudosubstrate domain, and induces a conformational change that is permissive for PDK docking to PRK. PDK subsequently phosphorylates the activation loop to render the kinase competent.

1.6.4 Biological functions

The micro-injection of kinase inactive form of PRK2 into NIH3T3 fibroblasts results in the disruption of actin stress fibers [82], while the kinase negative form of PRK1 prevented insulin-induced actin stress fiber breakdown and membrane ruffling [269], suggesting a role for PRK1 and 2 in regulating actin cytoskeletal reorganization. One of the cytoskeletal functions of PRK was elucidated from loss of function studies in the *Drosophila* PRK homologue (Prk). In the late stages of *Drosophila* development, dorsal closure takes place whereby cells of the lateral epidermis elongate along the dorsal-ventral axis. However, Prk loss of function mutants display a dorsal closure defect [81]. This dorsal closure defect appears to be highly similar to the phenotype of loss of function of Rho1 gene and several components of the Rac-mediated JNK cascade.

In *in vitro* experiments, PRK1 was shown to directly phosphorylate myosin and CPI-17 [50, 270]. Furthermore, PRK1 has been shown to interact with a number of

cytoskeletal proteins and thus may be involved in the modulation of cytoskeletal dynamics. PRK1 has been found to bind to the third spectrin-like repeats of the actin cross linking protein α-actinin [271]. PRK1 also directly binds to and efficiently phosphorylates the head-rod region of the intermediate filament proteins [272]. On the other hand, PRK2 has been reported to bind to tyrosine phosphatase-basophil like protein (PTP-BL), a large non-transmembrane protein tyrosine phosphatase implicated in the modulation of the cytoskeleton [273]. The binding and phosphorylation of a major cytoskeletal component by PRK1 and the strong colocalization of endogenous PTP-BL and PRK2 in lamellipodia-like structures, regions of extensive actin turnover, suggest the close correlation of PRK1 and 2 in the regulation of the actin-cytoskeleton.

Further biological roles of PRK may include vesicle and glucose transport. The overexpression of RhoA retards EGF receptor movements from early to late endosomal compartments after stimulation by EGF, and a catalytically inactive form of PRK1 mutant completely blocks this effect [274]. Thus PRK1 may regulate the kinetics of EGF receptor trafficking. In 3T3/L1 cells, expression of both Rho and PRK1 activates glucose transport, and replacement of dominant negative-form of Rho and PRK1 inhibits this. [275]. In rat adipocytes, transient expression of wild type PRK1 provokes an increase in the translocation of glucose transporter Glut4 to the plasma membrane. It remains to be determined whether PRK1 is involved in the transport of glucose under physiological conditions.

In addition, PRK2 may be involved in the regulation of apoptosis. PRK2 cleaved in the early stages of apoptosis binds to and prevents phosphorylation of PKB *in vivo* at

Ser 473 and Thr 308 [276]. Since phosphorylation of PKB is necessary for its full activation, PRK2 may inhibit PKB downstream signaling and abrogate its anti-apoptotic effects. In contrast, a recent study proposed a new function of PKC in cell death by demonstrating that PKC activation stabilizes X-linked inhibitor of apoptosis protein (XIAP) and thus suppresses tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) induced apoptosis [277]. PRK1 can be cleaved at specific sites in some apoptotic cells [278]. This cleavage seems to be catalyzed by caspase-3 or a related protease. The major proteolysis takes place between the N-terminal regulatory domain and the C-terminal catalytic domain, and generates a constitutively active kinase fragment which may contribute to signal transduction, eventually leading to apoptosis. Finally PRK1 may play some roles in the development of cancers, because PRK1 can interact with the E6 oncoprotein and phosphorylate E6 [279].

1.7 Approaches used to elucidate isozyme-specific functions of PKC

The discovery of staurosporine, an antifungal agent, as an inhibitor of PKC effective at nanomolar concentrations [280] led to development of many derivatives. Two such compounds, Ro31-8220 and Go6850 were subsequently used in cell-based assays to determine specific roles for PKCs. However, these were later shown to lack specificity and to inhibit several other protein kinases *in vitro* [281, 282]. One derivative of staurosporine, LY333531, which inhibits PKC-β more potently than other PKC isoforms, is reported to normalize the elevated levels of PKC activity in the retina and kidneys of diabetic rats [283]. It is in Phase III clinical trials for the treatment of diabetic microvascular disease.

In terms of basic science, kinase inhibitors are useful in elucidating downstream targets and signaling networks. Each kinase can have several substrates and be itself a substrate for other kinases. To delineate the complex network of kinase signaling pathways, matching of every kinase in a cell to specific phosphorylation sites on target proteins is necessary. Not every kinase will work in an *in vitro* assay and a peptide that is phosphorylated by a kinase *in vitro* does not necessarily reflect that kinase's physiological target. The holy grail of kinome analysis is to determine the physiological substrates of all kinases to begin to map out signaling pathways. In the absence of isotype-specific PKC inhibitors, it is difficult to assess the exact consequences of the activation of a certain isotype in terms of substrate phosphorylation and biological effect. This presents a major challenge at the moment, and several interesting models have been developed to address these issues.

For instance, a variety of stable cell lines have been generated that overexpress PKC isotypes [284, 285], allowing the examination of their effects on biological processes or specific proteins of interest. Although this approach is potentially fruitful, it has the drawback that effects of the overexpressed PKCs on protein phosphorylation may not be direct and may not be physiological.

The use of dominant negative mutants of PKC isotypes provides another way of assessing their functional involvement in signal transduction processes. These mutants are generated by modifying the ATP-binding site, thus abolishing the kinase activity of the enzyme. Overexpression of such kinase-negative mutants might lead to a disruption of the signaling pathways in which the kinase is normally active, by interacting with endogenous activators, activating kinases or binding proteins and so competing with endogenous wild-type protein. However, in this dominant negative

approach, it is unclear to what extent the kinase negative mutants affect signaling by other PKC isotypes. Thus it is difficult to assess the specificity.

1.7.1 PKC knock-out mice

A more direct approach for the study of isozyme-specific function of PKC isozymes is targeted gene inactivation (gene knock-out) in mice. Neural, immunologic and endocrine phenotypes have been reported.

Recently, the role of PKCα in primary T cell activation and tumor suppression was identified. Loss of PKCα had no apparent effect on T cell development and selection in the thymus. Instead, PKCα was required for the activation and *in vivo* function of mature $CD3^+$ T cells, such as recall antigen-induced T cell growth, IFN-γ production, and the support for class switching to IgG2a and IgG2b after OVA antigen challenge *in vivo*. The study indicated that, in addition to PKCθ, PKCα is part of a signaling pathway that is necessary for full antigen receptor-mediated T cell activation and T lymphocyte immunity [286]. While PKCα has been proposed to act both as a tumor promoter and a tumor suppressor in intestinal cancer via multiple signaling pathways [287], Oster and colleagues provided evidence that PKCα function as a tumor suppressor in intestinal cancer. Deletion of PKCα but not of *PKCζ* was found to promote polyp formation in wild-type mice and in the $Apc^{Min/+}$ tumor model [288]. Although PKCα appears to be one of the predominant isoenzymes in the kidney [289-291], studies by Yao et al showed that PKC-α appeared not to play a major role in renal sodium reabsorption but, consistent with its expression in the medullary collecting duct, contributed to urinary concentration in mice. Thus PKCα–/– mice showed approximately 50% greater urine flow rate and lower urinary osmolality

compared to PKCα +/+ mice and exhibited a modestly greater urinary flow rate and modestly lower urinary osmolality under basal conditions [292]. In addition, the hearts of Prkca-deficient mice were hypercontractile, whereas those of transgenic mice overexpressing Prkca were hypocontractile. Hence PKCα has been proposed to be a fundamental regulator of cardiac contractility and Ca^{2+} handling in myocytes. [293].

PKCβ mice were initially found to demonstrate deficiencies in B-cell function and impaired humoral immune responses [294]. Another study documented deficits in mast cell degranulation and interleukin-6 production [295]. It is not yet certain if both these changes were due to developmental effects of the null mutation or loss of enzyme in adult tissues. PKCβ-null mice have been found to show modest increases in insulin-stimulated translocation of GLUT-4 glucose transporters and in glucose transport in some tissues [296]. This can be rescued in part by transgenic expression of PKCβI, suggesting that it is due to the loss of that enzyme. Recent work had demonstrated PKCβ's contribution to the regulation of arterial blood pressure and its requirement for normal acid-base balance [292].

The T lymphocyte-restricted PKCθ is unique among other PKC members because it colocalizes with the T cell receptor in the T cell immunological synapse [297, 298]. The physiological functions of PKCθ for T cell receptor signaling were subsequently demonstrated by two independent studies with PKCθ-deficient mice [299, 300]. Peripheral T cells of $PKC\theta^{-/-}$ mice show reduced proliferation and IL-2 production and significant impairment in AP-1 and NF-κB activation [299, 300] as well as NFAT [300] activation in response to TCR/CD28 stimulation. Although studies have implicated the IκBα kinase IKK complex in linking PKCθ to NF-κB activation [301-

303], the pathway responsible for coupling PKCθ to AP-1 appears to be JNK independent [299]. However, another set of studies showed that at least in the immune response to murine gamma-herpesvirus 68 (MHV-68), PKCθ is not essential for the T-cell activation events leading to viral clearance [304]. This disparity in PKCθ function, where PKCθ is dispensable for certain T-cell functions during a viral infection *in vivo* but is required for T-cell activation *in vitro,* is currently unclear.

Studies have shown that local accumulation of fat metabolites inside skeletal muscle may activate a serine kinase cascade, involving serine phosphorylation of insulin receptor substrate–1 (IRS-1), leading to defects in insulin-mediated IRS-1–associated PI3K and glucose transport in skeletal muscle [305, 306]. Skeletal muscle insulin resistance is associated with increases in intramuscular fat metabolites (i.e., fatty acyl-CoA, diacylglycerol) and PKC-θ activity, suggesting an important role for PKC-θ in mediating fat-induced insulin resistance in skeletal muscle [307]. From PKC-θ null mice, it was demonstrated that PKCθ functions as a crucial component mediating fat-induced insulin resistance in skeletal muscle [308].

Studies from PKCλ knockout ES cells indicate that PKCλ is required for insulin-stimulated glucose transport. Insulin activated PKC-λ and stimulated glucose transport in wild-type (WT) PKC-$\lambda^{+/+}$, but not in knockout PKC-$\lambda^{-/-}$, ES cells. However, insulin-stimulated glucose transport was rescued by expression of WT PKC-λ in PKC-$\lambda^{-/-}$ ES cells [309].

Although PKCε null mice display normal responses to noxious thermal and mechanical stimuli, they show decreased nociceptor sensitization after local injection of epinephrine [310]. Similar findings were obtained in rats injected locally with a

peptide inhibitor of PKCε, confirming that responses observed in null mice are due to loss of PKCε function in mature neurons. In a different set of studies by the same laboratory, PKCε null mice were found to be supersensitive to the sedative effects of ethanol, barbiturates, and benzodiazepines and this correlates with enhanced sensitivity of γ-aminobutyric $acid_A$ receptors to the agonist properties of these drugs *in vitro* [311]. This supports other findings in rats and humans, indicating an inverse correlation between alcohol consumption and sensitivity to acute alcohol intoxication. These studies suggest that inhibition of PKCε may reduce alcohol consumption and prove useful in the treatment of alcoholism.

Recent work by Stawowy has demonstrated an essential role of PKCε for activation of β_1-integrins by angiotensin II (AII) in fibroblasts [312]. AII induces fibronectin, laminin and TGF-β_1 mRNA via its angiotensin type 1-receptor (AT_1) in cardiac fibroblasts [313, 314]. In PKCε (−/−) cells basal adhesion was not significantly disturbed, but AII treatment did not induce a significant increase of adhesion, in contrast to PKCε (+/+) cells where AII treatment induced a significant increase in adhesion to collagen I [312].

A brief period of ischemia-reperfusion protects myocardial tissue from a subsequent prolonged ischemia by delaying cardiomyocyte death, a phenomenon called ischemic preconditioning. Whereas the protective role of PKC-ε in preconditioning is well established, controversial results exist concerning the cardioprotective effects of PKC-δ [315, 316]. For instance, Wang et al. [317] demonstrated that mitochondrial translocation of PKC-δ is related to cardioprotection induced by ischemic preconditioning, whereas Fryer et al. [318] reported that PKC-δ plays an important

role in pharmacological-induced preconditioning but not in ischemic preconditioning. Yet another study, using metabolic and proteomic analysis, demostrated that preconditioning exaggerated ischemia-reperfusion injury in PKC-$\delta^{-/-}$ mice [319].

PKCγ null mice were first generated by Abeliovich et al [320]. Because this isozyme is exclusively expressed in the central nervous system, all phenotypes involved central nervous system function. The first one described was deficient in spatial learning, as observed during a Morris water maze probe test [320]. The deficit was mild and could be overcome by intensive training. It was associated with impaired contextual fear conditioning. Both findings suggest hippocampal dysfunction associated with impaired long-term potentiation in CA1 hippocampal pyramidal neurons after high-frequency stimulation of CA3 axons. A subsequent study by the same laboratory disclosed a second phenotype of gait ataxia associated with persistent multiple climbing fiber synapses on cerebellar Purkinje cells due to impaired synapse elimination during development [321]. Other laboratories have identified additional phenotypes in PKCγ mice. Malmberg et al found that these mice showed normal pain responses but decreased hyperalgesia after mechanical or inflammatory peripheral nerve injury [322]. Furthermore, nerve injury increases the levels of neuropeptide Y and neurokinin-1 receptor immunoreactivity and decreases substance P receptor immunoreactivity in the dorsal horn of the spinal cord, but these responses are diminished in PKCγ null mice. This findings suggest that PKCγ is important in the neural plasticity within the spinal cord after nerve injury that contributes to neuropathic pain [323].

It is interesting to note that while PKCs demonstrate relatively broad *in vitro* substrate specificity, the published PKC isozyme knockout studies underscore the specific roles played by each isozyme, indicating distinct *in vivo* functions. It is possible that the reason for the fairly benign phenotypes of the single PKC knockouts in mice is that other members of the same PKC subfamilies are able to substitute partially for the deleted gene. In this case, the crossing of single knockout mice to remove an entire PKC subfamily may give more definitive results.

Aim

This study seeks to determine the V5 domain as a determinant in isozyme-specific regulation. It aims to elucidate the contribution of the V5 domain to specific kinase function in PKCs, using PRK1 and PRK2 as a models. It is hoped that such studies lay the foundation for the design of isozyme-specific kinase inhibitor targeting the V5 domain. The diverse and distinct roles of individual PKC are, at least in part, attributed to differences in their structural features. The V5 region appears attractive for specific regulation due to its great variability in terms of amino acid length and sequence among the isozymes in the PKC family. While previous studies focused on the turn and hydrophobic motifs, this study concentrates on the function of the additional residues C-terminal to the absolutely conserved phenylalanine residue corresponding to the last residue in PKA (Phe^{350} in PKA, Phe^{939} in PRK1 and Phe^{977} in PRK2) in the regulation and in the establishment of isozyme-specificity of the PKC superfamily of kinases *in vivo*.

A further objective is to unravel the molecular instruction of the C-terminus of PKCs for PDK-1 interaction and subsequent priming reaction of the activation loop. In addition, the potential role of V5 in response to lipid activation, leading to the production of kinases with differential catalytic activity was examined. A final objective is to explain the functional difference of V5 deleted kinases by investigation of computer modeling of the three-dimensional structure of the catalytic domain of PRK1 and PRK2.

Consequently, this thesis aims to investigate and compare the role of the V5 domain in the regulation of two PKC superfamily isoforms, PRK1 and PRK2, by defining:

1) the structural elements within the V5 domain of PRK1 and PRK2 that are required for the catalytic competence of the kinases;
2) the necessity of the hydrophobic motif for PDK-1 interaction and activation;
3) the function of the C-terminal extension of PRK beyond the conserved phenylalanine (corresponding to Phe^{350} in PKA and Phe^{939} in PRK1 and Phe^{978}) in the response of the kinases to lipid activators;
4) differences in the contributions of V5 domain to the catalytic activity of PRK1 and PRK2

Chapter 2: Materials and Methods

2.1 MOLECULAR BIOLOGY

Table 2.1.1 Frequently Used Buffers and Media

TE	10 mM Tris/HCL pH 7.5 or pH 8.0, 1 mM EDTA (autoclaved)
50 X TAE	2 M Tris/Acetate, 0.1 M EDTA. The solution was adjusted to pH 7.7 with 0.2 M acetic acid.
10 X loading dye	0.1% bromophenol blue, 0.1% xylene cyanol, 40% glycerol
LB medium	1% bactotryptone, 0.5% yeast extract, 1 % NaCl (autoclaved)
2 X TY medium	1.6% bactotryptone, 2% yeast extract, 0.5% NaCl, pH 7.0 with NaOH (autoclaved)
Bacterial plates	1.5% bacto agar suspended in LB, or TY
Ampicillin	1000X stock of 50 mg/ml in water (sterilized by filtration)
5-bromo-4-chloro-3-indoyl-D-galactoside (X-gal)	20 mg/ml (2%) in dimethylformamide (DMF) stock (500X)
Isopropylthio-β-D-galactoside (IPTG)	1 M stock in water (2000X)
Ethidium bromide	10 mg/ml stock (20,000X)
Lysozyme	5 mg/ml stock
SOC	LB containing 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose
RF1	12g/L RbCl, 10g/L $MnCl_2$, 30 mM potassium acetate (pH 7.5), 1.5 g/L $CaCl_2.2H_2O$, 15% (v/v) glycerol. The solution was adjusted to pH 5.8 with 0.2 M acetic acid and filter sterilized.
RF2	1g/L RbCl, 10 mM MOPS (pH 6.8), 11g/L $CaCl_2.2H_2O$, 15% (v/v) glycerol. The solution was adjusted to pH 6.8 with 0.1 M NaOH and filter sterilized.
Pfu DNA polymerase 10 x reaction buffer	200 mM Tris-HCl (pH 8.8), 100 mM KCl, 100 mM $(NH_4)_2SO_4$

2.1.2 Vectors

Vectors used in this study are listed in Appendix A. pcDNA3 (Invitrogen) was used as an expression vector in mammalian cells. pGEX-kg (GE Healthcare, formerly Amersham Bioscience) was used for GST fusion protein expression in *E. coli*. Vectors were stored at -20^{o}C in TE buffer.

2.1.3 Plasmids

2.1.3.1 *Escherichia coli* (*E. coli)* strains

The *E. coli* strains and genotype used in the recombinant DNA experiments are as follows:

DH5α *supE44placU169(ΦlacZpM115) hsdR115 recA1 endA1 gyrA96 thi-1 relA1*

JM109 *recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiρ(lac-proAB) F'[traD36 proAB^{+} lacIq lacl^{q} lacZρM15]*

2.1.3.2 Gift plasmids

N terminally Myc-epitope-tagged rat PRK1 cDNA cloned into a pcDNA3 vector was a kind gift from R. E. H. Wettenhall (University of Melbourne). Human Myc-PRK2, Myc-RhoA-Q63L and C3 plasmids were a kind gift from P. J.Parker (Cancer Research UK, London). Human RhoA-T19N DNA and glutathione-S-transferase (GST)-tagged human wild-type RhoA DNA in a pGEX4T-3 vector were kind gifts from Silvio Gutkind (NIH) and R. M. Evans (Salk Institute), A. O. Brinkmann (University Medical Center Rotterdam, The Netherlands) respectively.

2.1.4 Polymerase chain reaction (PCR)

The PCR reaction was performed using a PTC-100 TM Programmable Thermal Controller, (MJ Research, Inc. Watertown, MA, USA). A PCR reaction mixture consisted of 100 ng of template DNA, 200 ng of each of the primers, 2 mM $MgCl_2$, 200 µM dNTPs, 1 x *Pfu* DNA polymerase reaction buffer and 2.5 U of *Pfu* DNA polymerase in a volume of 100 µl. The reaction was carried out for 30 cycles of 45 sec at 95 ^oC (denaturing), 30 sec at 55 ^oC (annealing), and 2 min/kb at 72 ^oC (extension) with one final cycle for 20 min at 72 ^oC.

2.1.5 Site directed mutagenesis using PCR

Circular plasmid DNA of about 8 kb in length was used a template. A pair of complementary primers was designed with the desired mutation in the middle of the primer sequence. Primer sequences used in this study to generate point and deletion mutants are listed in Appendix B. The reaction was carried out using *Pfu* Turbo for 16 cycles of 30 seconds at 95^oC, 30 seconds at 55 ^oC and 16 minutes at 68 ^oC. After the PCR, 5 µl of 3 M sodium acetate (pH 4.6) was added to the PCR mixture, followed by 100 µl of 100% ethanol and the DNA was precipitated at -70 ^oC overnight. The DNA was recovered at 14000 x *g* at 4 ^oC, washed with 70% ethanol and air-dried. The DNA pellet was dissolved in sterile water and digested with Dpn I at 37 ^oC for 5 h. Ethanol precipitation was then performed. The DNA pellet was dissolved in 10 µl of TE and transformed into *E. coli*. The mixture was plated onto LB-Ampillicin plates and the plates were incubated at 37 ^oC overnight. Single colonies were picked and grew in 5 ml of 2 X TY at 37 ^oC overnight. The DNA was extracted with the Wizard SV DNA purification system and the mutation was confirmed by sequencing with appropriate primers. These primers sequences are listed in Appendix C.

2.1.6 Precipitation of DNA

DNA was precipitated at -20 ^{o}C or -70 ^{o}C by addition of two volumes of absolute ethanol and sodium acetate (pH 5.2) to a final concentration of 0.3 M. The precipitated DNA was then pelleted by centrifugation at 14,000 × *g* for 30 minutes at 4 ^{o}C. The pellet was washed with 70 % ethanol (pre-cooled at -20 ^{o}C) and air-dried for 20 minutes. For the precipitation of DNA at low concentration (below 20 µg/ml), glycogen was added to a final concentration of 1 mg/ml as a carrier to facilitate efficient precipitation.

2.1.7 Transformation of *E. coli*

2.1.7.1 Preparation of competent cells for transformation by heat shock

1 litre of LB was inoculated with 10 ml of an overnight culture of DH5α (F') and grown at 37 ^{o}C in a culture shaker until in log phase, OD_{600} approximately 0.5-0.6. The flask was chilled on ice for 15-30 minutes and the cells pelleted by centrifugation in a chilled rotor for 5 min at 2,000 x *g*. After all of the supernatant was discarded, the bacterial pellet was washed twice by resuspension and then pelleted in 1L of cold deionised water in a chilled rotor for 5 min at 2,000 x *g*. After pelleting the washed bacteria the supernatant was removed and the cells resuspended in 30 ml of cold 50 mM $CaCl_2$, 10 mM NaCl, 10 mM $MgCl_2$. the cells were repelleted by centrifugation at 3,020 x *g*. The supernatant was removed and the cells then resuspended in 2-3 ml of 50 mM $CaCl_2$, 10 mM NaCl, 10 mM $MgCl_2$ and 10% glycerol, snap-frozen in liquid nitrogen at 100 µl aliquots in Eppendorf tubes and stored at -80 ^{o}C.

2.1.7.2 Heat shock transformation of *E. coli*

Competent cells were thawed on ice, and 5-10 µl of a ligation mix was added. After a gentle mix the DNA-cells mixture was incubated on ice for 30 min and then heat shocked in a 42 ^{o}C water bath for 5 min. The cells were then transferred back to ice and allowed to recover for 20 min. Subsequently, 1 ml of LB media was added and the cells allowed to express their antibiotic resistance for 1 h at 37 ^{o}C in an orbital shaker set at 150 rpm. After this incubation, the cells were pelleted by centrifugation and resuspended in 200 µl of LB media for plating on selective media.

2.1.8 Plasmid DNA preparation

2.1.8.1 DNA Minipreps using the boiling method.

An overnight culture (1.5 ml) was pelleted in a microfuge tube and the supernatant discarded. The bacterial pellet was then resuspended by vortexing in 300 µl of STET buffer before adding lysozyme solution to a final concentration of 30 µg/ml in each tube. The samples were then boiled for 10 min in a heating block after which they were centrifuged at 12,000 x *g* in a benchtop centrifuge for 10 min to pellet the debris. The white gelatinous pellet of bacterial debris was removed from each tube with either a sterile toothpick or a sterile pipette tip. An equal volume of isopropanol was added to the supernatant to precipitate the DNA and the tubes mixed by gently inverting several times. The precipitated DNA was then pelleted by centrifuging at 12,000 x *g* for 10 min after which the pellet was resuspend in 50 µl of TE with DNAse-free RNAase added and two µl of the final suspension was used for restriction analysis.

2.1.8.2 High Quality Minipreps

Small-scale preparation of plasmid DNA, or minipreps, for small volumes of bacterial cultures (1-3ml) were carried out using the Hybaid Spin prep system as described in the manufacturer's handbook. This method is based on alkaline lysis of the bacterial cells and precipitation of debris, followed by a purification of plasmid DNA using a silica resin based on differential binding of the DNA to the silica at different pH/ionic strengths.

2.1.8.3 Qiagen maxi-preps

Large-scale preparations of plasmid DNA, or maxipreps, of large culture volumes (0.5-1.5 L) were performed with the Qiagen Plasmid Maxi Kit using Qiagen-tip 500 resin columns according to the manufacturer's protocol. This plasmid DNA purification technique is also based on an alkaline lysis procedure and subsequent purification of DNA using an ion exchange resin.

2.1.9 Agarose gel electrophoresis of DNA

Analysis of plasmid and DNA fragments from 0.2 to 8 kb in size was carried out by electrophoresis on horizontal agarose gels. Gels contain 0.8 to 1.5% (w/v) electrophoresis grade agarose in TAE or TBE buffer and GelStar® Nucleic Acid Gel Stain (Cambrex Bio Science Rockland, Inc #50535, stock supplied at 10,000 X concentration). Molten agarose was cooled to 55 ^{o}C and poured into a sealed horizontal gel casting tray with a comb positioned. The polymerized gel was subsequently submerged in an electrophoresis tank containing TAE buffer.

DNA samples were mixed with 6 X DNA loading dye before loading into the wells. Electrophoresis was performed at a constant voltage of 9 V/cm. The migration of DNA was monitored by the migration of the bromophenol blue dye in the loading buffer and DNA bands were visualized under UV light and image captured using the LAS imager.

2.1.9.1 Isolation of DNA fragments from agarose gels

Linearised DNA fragments that had been separated on the basis of size were visualized by staining with GelStar®. Desired bands of the appropriate size were excised from agarose gels using a clean scalpel and placed into clean 1.5 ml Eppendorf tubes. DNA was extracted from the agarose slice using the QIAquick-Agarose Gel Extraction kit from Qiagen which is based on solubilisation of the agarose with a chaotropic agent and mild heat and binding of DNA to a silica based resin. DNA isolation was carried out according to the manufacturer's guidelines.

2.1.10 DNA Sequencing using the BigDye Terminator cycle sequencing system

DNA sequencing was carried out with BigDye Terminator Cycle Sequencing system (Perkin-Elmer, ABI Prism377). This method is based on the dideoxy chain-termination protocol originally described in Sanger et al. [324], but uses dye-labeled terminators for a rapid DNA sequence determination. Fluorescent dye terminators attached to a dideoxynucleotide via a linker allow single-tube reaction set-up, longer reads, reduced pipetting and liquid handling, as well as increased sample throughput. Such methods routinely generate 600-800 bp of sequence.

Both the ABI Prism™ dRhodamine and BigDye™ Terminator cycle sequencing system (Perkin-Elmer) use these principles of fluorescent automated DNA sequencing. The dichlororhodamine dyes (ABI Prism dRhodamines) are an improvement over conventional rhodamine dyes. dRhodamines are better spectrally resolved and their sequencing products show reduced background noise. This results in cleaner signal and greater base calling accuracy at longer readlengths. The BigDye Terminator is a further improvement over the dRhodamine dyes. BigDye terminators contain a fluorescein energy donor linked to a dRhodamine acceptor dye by a highly efficient energy transfer linker. This allows for very efficient energy transfer in a single dye molecule. Hence, brighter and cleaner signals are obtained.

In the BigDye Terminator cycle sequencing systems, the dye terminators, dNTPs, AmpliTaq® DNA polymerase FS sequencing enzyme and reaction buffer are premixed in a single tube (Terminator Ready Reaction Mix). The reaction was performed using 4 µl of the Terminator Ready Reaction Mix, DNA template (250 ng double-stranded DNA), and 1.6 pmole primer in a 10 µl total volume. The thermal cycle profile of the 30 cycles reaction consist of denaturation at 96 ^{o}C for 10 s, annealing at 50 ^{o}C for 5 s and extension at 60 ^{o}C for 4 min. After the reaction, the products was precipitated with 10 µl of deionized water, 2 µl of 3 M sodium acetate (pH 4.6) and 50 µl of 95 % ethanol, to remove excess dye terminators. The DNA was pelleted by centrifugation at 14,000 x *g* for 30 min at 4 ^{o}C. The pellet was then rinsed twice with 70 % ethanol and vacuum dried. Prior to loading of sample, the pellet was resuspended in 4 µl of loading buffer (5 volumes of deionized formamide and 1 volume of 25 mM EDTA, pH 8.0, with 50 mg/ml blue dextran). The samples were denatured at 95 ^{o}C for 2 min and loaded onto a vertical gel system. The samples were

electrophoresed on the ABI PRISM® 377 DNA Sequencer (Perkin-Elmer) which is linked to a computer for automatic analysis of the multiple fluorescent dyes labeled DNA molecules.

2.2 Protein Analysis

Table 2.2.1 Buffers for protein analysis

2 X SDS gel-loading buffer	100 mM Tris HCl pH 6.8, 200 mM dithiothreitol (DTT), 4% SDS, bromophenol blue, 20% glycerol.
10 X Tris-glycine electrophoresis (PAGE) buffer, pH 8.3	30.2 g Tris, 188 g glycine, 50 ml 20% SDS, add distilled water to 1000 ml
Resolving gels for Tris-glycine SDS-Polyacrylamide Gel Electrophoresis (PAGE)	10% (w/v) acrylamide, 0.37 M Tris (pH 8.8), 0.1% (w/v) SDS, 0.4% (w/v) ammonium persulfate (APS), 0.04% (w/v) TEMED.
5 % stacking gels for PAGE	5% (w/v) acrylamide, 62.5 mM Tris (pH 6.8), 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.1% (w/v) TEMED.
Western blot transfer buffer, pH 8.3	25 mM Tris, 192 mM glycine, 20% (v/v) methanol (do not adjust the pH).
TBST buffer	100 mM Tris, 1.5M NaCl, 0.1% Tween-20 (v/v)
Cell lysis buffer (Buffer A)	20 mM Tris pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% (v/v) NP-40, 20 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium vanadate, 10% (v/v) glycerol. Add Protease Inhibitor cocktail just before use (from Roche Molecular Biochemicals/Boehringer, catalogue No. 1836145)

2.2.2 Sample preparation

Cells were washed with ice-cold PBS and lysed in the wells by adding 200 µl of ice-cold Buffer A. The plate was incubated in 4^{o}C for 15 min. The lysate was collected and centrifuged at 12000 x *g* for 30 min at 4^{o}C. The clarified supernatant was separated from the pellet. SDS loading buffer was added to the remaining pellet and the mixture was subjected to one cycle of boiling and chilling on ice for 3 min each to denature the DNA. SDS loading buffer was also added to the supernatant and both pellet and supernatant fractions were boiled for 5 min. The samples were further clarified by centrifugation at 12,000 x *g* for 15-30 min at room temperature before undergoing SDS-PAGE.

2.2.3 Preparation of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

A standard discontinuous SDS-PAGE gel is made up of two layers: a stacking gel on the top and a resolving gel at the bottom of the gel. The composition of the stacking gel is 4 % (w/v) acrylamide-bisacrylamide, 0.1 % (w/v) SDS, 0.05 % (w/v) ammonium persulfate, 0.1 % (w/v) TEMED, 125 mM Tris-HCl, pH 6.8.

In this study, 7.5 %, 10 % and 12.5 % resolving gels were used to resolve proteins of various molecular weights. Ten percent gels were used in most experiments. The composition of the 10 % resolving gel was 10 % (w/v) acrylamide-bisacrylamide, 0.1 % (w/v) SDS, 0.4 % (w/v) ammonium persulfate, 0.04 % (w/v) TEMED, 375 mM Tris-HCl, pH 8.8.

The Bio-Rad Protean-III gel apparatus was set up to cast the SDS-PAGE gel. The resolving gel was poured first and a layer of butanol was added on top to erase the bubble and make the liquid interface horizontal. After the resolving gel was set (this normally took 40 min at room temperature), butanol was removed. The stacking gel solution was pipetted onto the top of the resolving gel and the wells were prepared by inserting the comb into the stacking gel. After the gel was set, the whole gel set was submerged into the electrode buffer (25 mM Tris-base, 250 mM glycine, 0.1 % (w/v) SDS). The wells were flushed using the electrode buffer. Samples were loaded and the electrophoresis was carried out at a constant voltage of 100 V for 15 min to ensure that the dye front reached the interface of the stacking gel and the resolving gel. Electrophoresis was continued at 180 V until the dye front reached the edge of the gel.

2.2.4 Gel staining (Coomassie blue staining of the SDS-PAGE gel)

After electrophoresis, the gel was peeled off from the plate and soaked in staining solution (0.25 g Coomassie brilliant blue R250, 45 ml methanol, 45 ml H_2O, and 10 ml glacial acetic acid) at room temperature with gentle agitation for 1 hour. The staining solution was removed and destaining solution (40 % methanol and 10 % acetic acid) was added. According to the visibility of the protein bands, the destaining procedure lasted from a few hours to overnight.

2.2.5 Western Blotting

Protein samples were electrophoresed on SDS-PAGE gel. Nitrocellulose membrane (PROTRAN, #10401196 from Schleicher & Schuell) was cut to cover the gel. Two sheets of 3 mm paper were also prepared and soaked in cold transfer buffer (25 mM Tris-base, 192 mM glycine, 20 % (v/v) methanol). The whole set was assembled on the blotting panel in the following order: Scotchbrite, 3 mm paper, gel, nitrocellulose

membrane, 3 mm paper and Scotchbrite. When nitrocellulose membrane was placed on the gel, air bubbles were rolled out. The blotting assembly was then secured in the blotting apparatus that was filled with pre-cooled (4 ^{o}C) transfer buffer and electrotransfer carried out at 100 V for 1 h.

At the end of blotting, the apparatus was dis-assembled and the nitrocellulose sheet placed in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 % (v/v) Tween-20). The membrane was blocked with 10 ml of blocking solution (TBST with 5 % skim milk) for 60 min. The membrane was incubated in the primary antibody for 1 h with gentle agitation at room temperature. The membrane was washed three times with TBST for 5 min each. The secondary antibody was diluted 50,000 fold in a volume of 25 ml and the membrane incubated in this for 1 h. The membrane was washed 3 times with TBST, for 5 minutes each. Chemiluminescence solution (SuperSignal West Dura chemiluminescence, PIERCE, #34075) was added (200 µl/ each membrane for a mini gel) to the membrane and the chemiluminescence was detected using a LAS-1000 plus Chemiluminescence and Fluorescence Imaging System (Fuji Film) or Kodak BioMax MR films.

2.2.5.1 Sources of antibodies

Sigma-Aldrich

Anti-PKC-ζ, #P0713, rabbit polyclonal; anti-PDK1, #P3110, rabbit polyclonal; anti-FLAG M2, # F3165, mouse monoclonal; anti-β-actin, #A5441, mouse monoclonal.

Cell Signaling

Anti-Phosphor-(Thr) PDK1 Substrate Antibodies, #2291, rabbit polyclonal; anti-PKD/PKCµ, #2052, rabbit polyclonal; anti-PDK1, #3062, rabbit polyclonal; anti-Phosphor-PRK1 (Thr778)/PRK2 (Thr816), #2611, rabbit polyclonal.

Santa Cruz Biotechnology

Anti-RhoA (26C4), sc-418, rabbit polyclonal.

Roche Applied Science

Anti-Myc, clone 9E10, #1667203, mouse monoclonal.

Secondary antibodies (from Pierce Biotechnology)

ImmunoPure Goat anti-Mouse IgG, (H+L), peroxidase conjugated, #31430; ImmunoPure Goat anti-Rabbit IgG, (H+L), peroxidase conjugated, #31210.

2.2.6 Immunoprecipitation (IP)

2.2.6.1 Magnetic beads coating

For one million cells lysed by Buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1 % NP-40, 20 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium vanadate, 10 % glycerol, supplemented with protease inhibitor cocktail and 1 μM okadaic acid), 8 μl of paramagnetic Dynabeads were used to pull down the proteins for IP. The beads coated with antibodies against mouse IgG1 (DYNAL, #110.12) or rabbit IgG (DYNAL, #112.04) were washed by vortexing in washing buffer (5 % serum in PBS (phosphate-buffered saline, 140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4 \cdot 2H_2O$, 1.8 mM KH_2PO_4, adjusted to pH 7.4)) at 4 ^oC. The beads were collected by a magnetic bar and blocked with COS1 cell homogenate supplemented with 20% serum for 1 h by gentle rotation at 4 ^oC. The primary antibody (0.5 μg/ 8 μl beads) was added in 500 μl washing buffer containing the beads followed by incubation overnight at 4 ^oC with gentle rocking. Before immunoprecipitation, the beads were washed 3 times for 5 min each using PBS containing 5 % calf serum at 4 ^oC with gentle rocking. Finally, the antibody-coated beads were resuspended in PBS containing 5 % calf serum.

2.2.6.2 Immunoprecipitation

Cultured cells were lysed in Buffer A. The clarified cell lysates were incubated with paramagnetic Dynabeads that had been coated with relevant primary antibodies overnight at 4 ^{o}C. The immunoprecipitated complexes were washed twice with 1 ml buffer B (1 % NP-40 in 1 XPBS, 1 mM PMSF (phenylmethylsufonyl fluoride) added before use) for 30 min, twice with buffer C (10 mM Tris-HCl, pH 7.5, 0.5 M LiCl) for 30 min with gentle rotation. For kinase assay, the immunoprecipitates were subjected to two more washes in buffer D (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl). For co-immunoprecipitation, the complexes were washed twice with 1 ml buffer B and twice with buffer F (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 300 mM NaCl).

2.2.7 Preparation of GST-RhoA and GST-Tau1

GST-RhoA and GST-Tau1 plasmids were transformed separately into *E. coli* strain BL21-CodonPlus (Stratagene, La Jolla, CA). A single colony was picked and cultured in 10 ml of LB medium (10 g bacto-trypton, 5 g yeast extract, 10 g NaCl in 1 liter, pH 7.0) with 100 μg/ml ampicillin and 20 μg/ml chloramphenicol overnight at 37 ^{o}C. The *E. coli* culture was then added to 100 ml LB medium and incubated for another 16-18 h. The total of 100 ml of medium with the cells was transferred to fresh 900 ml pre-warmed LB medium and incubated at 37 ^{o}C with shaking for 1 h. IPTG (isopropylthio-β-D-galactoside) was added to the bacterial culture upon reaching an $OD_{600\ nm}$ of 0.6~0.7 to a final concentration of 0.1 mM to induce the expression of GST-RhoA. Three hours later, *E. coli* cells were pelleted by centrifugation at 1935 × *g* (Beckman J2-MC centrifuge, JA-20 rotor) for 10 min at 4 ^{o}C and resuspended in 3 ml of cold lysis buffer (50 mM Tris-HCl, pH 7.6, 50 mM

NaCl, 25 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 1 % Triton X-100) on ice. The bacteria were sonicated to release intracellular proteins. The sonicate was cleared by centrifugation at 17,300 × *g* for 10 min at 4 ^{o}C. The supernatant was removed for column purification of GST-tagged proteins with a 20 µl aliquot of the supernatant set aside for Western blot analysis. The commercial glutathione-agarose beads (Sigma, #G4510) were washed once with distilled water and followed by 3 volumes of lysis buffer to remove ethanol (used as a preservative for the column). The washed beads were resuspended in lysis buffer as a 1:1 suspension (v/v). One ml of the suspension was added to the supernatant of the *E. coli* sonicate. The mixture was incubated at 4 ^{o}C for 1.5 hour with gentle rotation. The agarose beads were pelleted by centrifugation in a benchtop centrifuge at 1000 × *g* for 1 min and washed with 5 ml cold lysis buffer (without DTT and PMSF) 5 times. GST-tagged proteins was eluted with 0.5 ml of freshly prepared elution buffer with 5 mM reduced glutathione (pH 7.5) (Sigma, #G4251) with gentle rotation for 20 minutes at 4 ^{o}C. The beads were regenerated and aliquots of GST-RhoA and other fractions were subjected to gel staining to verify the purity of the eluted GST-RhoA.

2.2.8 GTP-γ-S loading of RhoA

Purified GST-RhoA (7.8 µM in 27 µl) was loaded with 30 µM GTP-γ-S (Sigma, # G8634) at 30 ^{o}C for 20 minutes in loading buffer (0.05 M Tris-HCl, pH7.5, 1 mM EDTA, 5 mM $MgCl_2$, 0.06 % CHAPS). Immediately after loading, the entire mixture of GTP-γ-S-loaded RhoA was mixed with immunoprecipitated WT-PRK1 (wild type) and various truncation mutants and incubated at 4 ^{o}C for 1 h with rocking. The immune-complex was then washed twice with buffer A for 30 min. Half of the complex was subjected to Western blot analysis; while the other half of the beads was

subjected to kinase assay. Autophosphorylation was performed for WT-PRK1 and various mutants followed by phosphorimaging and Western blotting.

2.3 Cell Culture and Transfection

2.3.1 Cell lines

COS1, African green monkey kidney fibroblast, ATCC #CRL-1650;

HEK293, human fetus kidney epithelial, ATCC #CRL-1573;

NIH3T3, mouse embryo fibroblast, ATCC #CRL-1658;

N1E-115, mouse neuroblastoma C-1300, Passage No.#17, ATCC, #CRL-2263.

2.3.2 Cell culture medium

Routine cell culture was carried out in a medium of 90 % high glucose (4.5 g/L) version of DMEM (Invitrogen, #12100-046) supplemented with 10 % fetal calf serum and 2 mM L-alanyl-L-glutamine (Invitrogen, #35050-061).

2.3.3 Transfection by liposomal method

2.3.3.1 Transfection of mammalian cells using LIPOFECTAMINE Reagent

Cells were seeded at 3×10^5 per well in a 6-well plate one day prior to transfection. Cells of 60-80 % confluency were transfected. One hundred µl of OPTI-MEM (Invitrogen, #22600-050) was mixed with 6 µl LIPOFECTAMINE Reagent (Invitrogen, #18324-012). At the same time, 100 µl of OPTI-MEM was mixed with 1 µg of plasmid. These two mixtures were combined and incubated at room temperature for 45 min. Eight hundred µl of serum–free medium was added to the mixture and the mixture was transferred to cells in the wells which were pre-washed with PBS. The cells were incubated at 37 ^oC in a humidified atmosphere with 5 % CO_2 and 95% air.

Six hours later the transfection mixture was removed and replaced with the complete medium containing 10 % fetal bovine serum.

2.3.3.2 Transfection of mammalian cells with PolyFect Reagent

HEK 293 cells or CHO-K1 cells were cultured in 60-mm plates with 4 ml of DMEM supplemented with 10 % FCS (Fetal calf Serum). Sixteen hours prior to transfection, cells were seeded at a density of 8×10^5 cells per plate. One hundred and fifty µl of OPTIMUM medium containing 2.5 µg of DNA for CHO-K1 cells (4 µg for HEK293 cells) was mixed with 15 µl of PolyFect Transfection reagent (QIAGEN, # 301107) for CHO-K1 cells or 40 µl reagents for HEK293 cells for 10 min at room temperature to allow the formation of transfection complex. Three ml of the full growth for CHO-K1 cells or low-serum medium (DMEM with 0.5 % FCS) for HEK 293 cells was added to the cells and 1 ml of the same medium was added to the tube containing the transfection complex. The mixture was transferred to plates with cells and the cells were incubated with the complexes for at least 24 h prior to other assays.

2.4 Assays

2.4.1 *In vitro* kinase assay (immune-complex kinase assay)

2.4.1.1 Autophosphorylation

Immunoprecipitates were used in immune-complex kinase assays carried out in 30 µl of reaction mixture containing buffer (20 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 40 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) and 1 µl of protease inhibitor cocktail (Roche Diagnostics, Protease Inhibitor Cocktail Tablets, #1697498) and 200 µM γ-[^{32}P]-ATP (GE Healtcare #AA0018, specific activity 360 cpm/pmol) at 30 ^{o}C for 7 min. The solution was mixed by tapping every 2 min and, after 7 min, SDS loading dye (50 mM Tris-HCl, pH 6.8, 14.4 mM mercaptoethanol, 2 % (w/v) SDS, 0.05 %

(w/v) bromophenol blue, 10 % (w/v) glycerol) was added to the mixture to stop the reaction. The phosphorylated proteins were separated by 10 % SDS-PAGE and analyzed using a BAS-2500 phosphorimager (Fuji Photo Film) and Image Gauge 4.0 software. The kinase activity of each sample was corrected for background phosphorimaging densitometric units from negative controls of vector transfected cells. The amount of kinase in each sample was determined by chemiluminescence-based Western blotting using a LAS-1000plus Imaging System equipped with a CCD camera that does not register radiation from ^{32}P. The relative intensity of the kinase in question was then used to normalize the phosphorimaging densitometric units to derive the specific activity of the kinase.

2.4.1.2 Transphosphorylation

After immunoprecipitation and washing, 30 µl of kinase buffer (20 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 40 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) including 110 µM S6 peptide, which is based on human ribosomal protein S6 residues 229-239 (AKRRRLSSLRA-NH_2), 1 µl of protease inhibitor cocktail and 200 µM γ-[^{32}P]-ATP (specific activity 360 cpm/pmol) were added to the immunoprecipitates. The transphosphorylation reaction was carried out for 7 minutes at 30 ^{o}C and terminated by adding 3.3 µl stop solution (200 mM ATP, 500 mM EDTA, pH 8.0). The kinase bound to Dynabeads was retained at the bottom of the tube by a magnet. The supernatant containing the peptide substrate was transferred onto P81 phosphocellulose paper (Whatman, #3698021). The paper was washed three times with 75 mM phosphoric acid and once with 95% ethanol and 5% H_2O and dried. The incorporated radioactivity was determined by scintillation counting. The kinase activity of each sample was corrected for background counts from negative controls

of vector transfected cells. The amount of kinase in each sample was determined by chemiluminescence-based Western blotting using a LAS-1000plus Imaging System equipped with a CCD camera that does not register radiation from ^{32}P. The relative intensity of the kinase was then used to normalize the counts to derive the specific activity of PRK1.

2.4.2 Protein half-life assay

Twenty hours post-transfection, the medium for COS-1 cells was replaced with a fresh medium containing a final concentration of 200 μg/ml cycloheximide. In these experiments, the cycloheximide used was from a stock solution of cycloheximide that was dissolved in tissue culture grade dimethyl sulphoxide (Sigma, catalogue numbers C7698). At the time points indicated, cells were lysed with buffer A and the lysate was centrifuged at 14,000 x *g* for 30 min to obtain a clarified lysate and a pellet which corresponds to the detergent soluble and –insoluble fractions of the lysate. The zero point of the time course was defined as 4 h after the addition of cycloheximide. During the course of experimentation, the culture medium was replaced every 8 h with freshly prepared medium containing cycloheximide. The amounts of exogenous kinase in each sample were normalised by that of endogenous β-actin in Western blotting.

2.4.3 N1E-115 neurite retraction assay

N1E-115 neuroblastoma cells were cultured in DMEM supplemented with 10 % FCS. One day prior to transfection, cells were seeded in 24-well plates at a density of 5×10^4 cells /well. Cells were transfected with WT-PRK1, empty vector (pcDNA3), PRK1-S377A, RhoA-T19N, WT-RhoA, WT-PRK1 plus RhoA-N19, WT-RhoA plus

RhoA-T19N, C3 (exoenzyme, ADP-ribosyltransferase) and C3 plus WT-PRK1 using LIPOFECTAMINE reagent and incubated in serum-free medium for 30 h to induce neurite outgrowth. Cells were treated with 3 μM LPA for 3 min and photographed immediately using an Axiovert-25 inverted phase contrast microscope (Carl Zeiss) equipped with a Coolpix 990 CCD camera (Nikon). At least one hundred cells for each well were counted. The morphology of cells was classified as round: rounded cells without neurites; flat: flattened cells without neurites or extensions shorter than the soma diameter; and neurites: flattened cells with at least one process greater than the soma diameter.

2.5 Homology modeling

A structural alignment of human PKCθ at 2.0Å (PDB code 1XJD [87]), human PKBβ at 1.6Å (PDB code 1O6L [85]), the catalytic subunit of PRK1 at 2.04Å and the turn/hydrophobic motif from the recently solved structure of human PKCι at 3.0Å [325] was generated by superposing the 3-D coordinates according to a CLUSTALW [87] alignment of their sequences, followed by manual manipulation using QUANTA (Accelrys, San Diego, CA, USA). To this alignment, the sequence for the catalytic domains of PRK1 (residues 612-946) was added and aligned. Insertions and deletions relative to the known structures were positioned in the loop regions, keeping the secondary structure elements intact. The program MODELLER [326] was then used to generate 20 initial homology models of bovine $PRK1_{612-946}$ based on this alignment. The model with the lowest objective function was chosen as the representative model for further study. To provide some structural basis for the positioning of the final nine residues, the PRK1 sequence was also used to search the PDB using LOOPP [327] and BLAST [328]. The models were then further optimized

using CHARMM [329] to add hydrogen atoms to the protein, and phosphate atoms to the known phosphorylated residues, followed by a restrained energy minimization to remove any remaining steric clashes.

2.5.1 Molecular dynamics simulation of PRK1 model

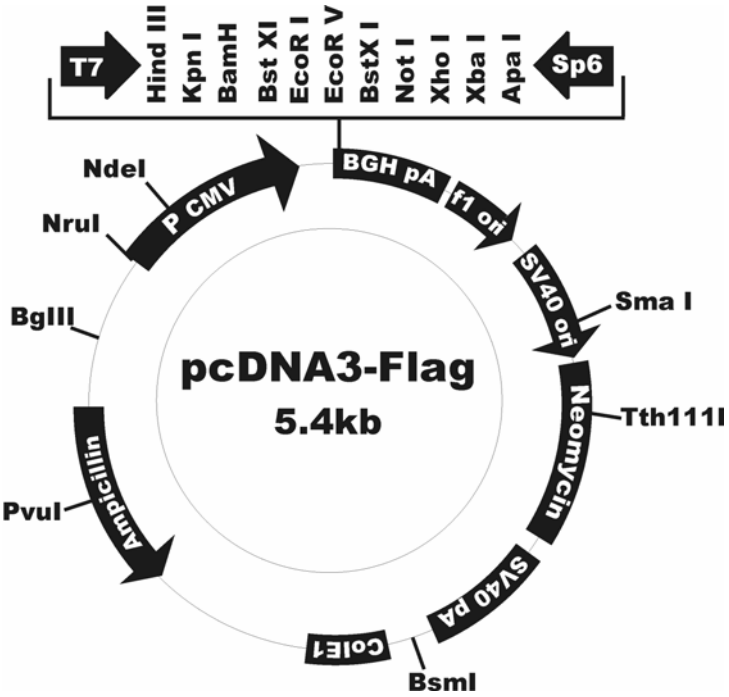
To probe the possibility of structural changes in the native and mutant models, the models structures were subjected to a 10 ns molecular dynamics simulation using NAMD software with the CHARMM22 force field.

The model structures were solvated to a distance of eight Å from all protein atoms in a rectangular box of water. Fifteen sodium cations were added to each box to neutralize the overall negative charge of the structures. Molecular dynamics simulations were performed with a 2 fs time step at a constant temperature of 300 K and a constant pressure of one atmosphere under periodic boundary conditions. The Particle Mesh Ewald method was used for electrostatics, and a 12 Å cut-off was used for van der Waals interactions. The TIP3P water model was used to model the solvent.

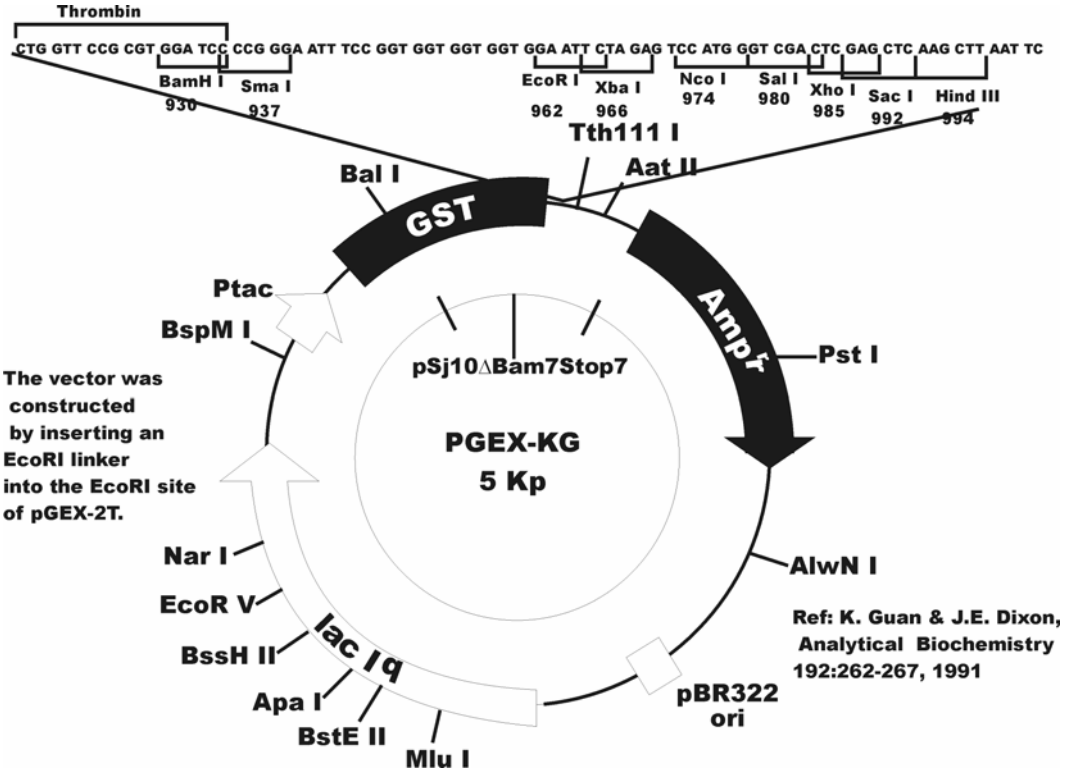
The ensemble was minimized for 1500 steps using the conjugate gradient method. This was followed by a heating protocol where the ensemble temperature was increased from 0 K to 300 K over 5 ps. Afterwards, the backbone atoms of the structure were fixed, while the side chains and solvent were allowed to move unrestrained for a further 30 ps, followed by totally unrestrained equilibration for 10 ps. The production run was then performed for 10 ns, with the SHAKE algorithm implemented to restrain all hydrogen motions. Coordinates were saved every 1 ps.

Appendix A: Vectors

pcDNA3



pGEX-kg



Appendix B: Primer sequences

Rat PRK Δ946

DWS 240 (sense)
5'- GTG GCA GGA GGC TAA TAG CCC TAA GC -3'

DWS 241 (antisense)
5'- GC TTA GGG CTA TTA GCC TCC TGC CAC -3'

Rat PRK Δ944

DWS 238 (sense)
5'- GAC TTT GTG GCA TGA GGC TAT TAG C -3'

DWS 239 (antisense)
5'- G CTA ATA GCC TCA TGC CAC AAA GTC -3'

Rat PRK Δ942

DWS 236 (sense)
5'- GAT TTC GAC TTT TAG GCA GGA GGC -3'

DWS 237 (antisense)
5'- GCC TCC TGC CTA AAA GTC GAA ATC -3'

Rat PRK Δ941

DWS 1095 (sense)
5'- CGG GAT TTC GAC TAA GTG GCA GGA GGC TAT TAG -3'

DWS 1096 (antisense)
5'- CTA ATA GCC TCC TGC CAC TTA GTC GAA ATC CCG -3'

Rat PRK Δ940

DWS 234 (sense)
5'- C CGG GAT TTC TAA TTT GTG GCA GGA G -3'

DWS 235 (antisense)
5'- C TCC TGC CAC AAA TTA GAA ATC CCG G -5'

Rat PRK Δ939

DWS 244 (sense)
5'- CC TTC CGG GAT TGA GAC TTT GTG GCA G -3'

DWS 245 (antisense)
5'- C TGC CAC AAA GTC TCA ATC CCG GAA GG -3'

Rat PRK Δ938

DWS 232 (sense)
5'- G GCC TTC CGG TAA TTC GAC TTT GTG -3'

DWS 233 (antisense)
5'- CAC AAA GTC GAA TTA CCG GAA GGC C -3'

Rat PRK Δ924

DWS 431 (sense)
5’- G AGC CCT CCT CGG TAA GCA CGG CCC -3’

DWS 432 (antisense)
5’- GGG CCG TGC TTA CCG AGG AGG GCT C -3’

Rat PRK Δ923

DWS 429 (sense)
5’- G AGC CCT CCC TGA GAT GCA CGG -3’

DWS 430 (antisense)
5’- CCG TGC ATC TCA GGG AGG GCT C -3’

Rat PRK Δ921

DWS 479 (sense)
5’- CC ACA CTG AGC TAG CCC CGG GAT GC -3’

DWS 480 (antisense)
5’- GC ATC CCG GGG CTA GCT CAG TGT GG -3’

Rat PRK Δ920

DWS 367 (sense)
5’- C CCC ACA CTG TGA CCT CCA CGG GAT G -3’

DWS 368 (antisense)
5’- C ATC CCG TGG AGG TCA CAG TGT GGG G -3’

Rat PRK Δ918

DWS 365 (sense)
5’- GCC CCC TAA CTG AGC CCT CCC CGG -3’

DWS 366 (antisense)
5’- CCG GGG AGG GCT CAG TTA GGG GGC -3’

Rat PRK Δ885

DWS 205 (sense)
5'- G GAC TGG GAT TGA CTG CTG GCC -3'

DWS 206 (antisense)
5'- GGC CAG CAG TCA ATC CCA GTC C -3'

Rat PRK Δ882

DWS 207 (sense)
5’- C AGG ACT CTG TAG TGG GAT GCC C -3’

DWS 208 (antisense)
5’- G GGC ATC CCA CTA CAG AGT CCT G -3’

Rat PRK Δ881

DWS 363 (sense)
5’- C TTC AGG ACT TAG GAC TGG GAT GCC -3’

DWS 363 (antisense)
5’- GGC ATC CCA GTC CTA AGT CCT GAA G -3’

Rat PRK Δ879

DWS 425 (sense)
5’- G CCT TTC TTC TGA ACT CTG GAC TGG -3’

DWS 426 (antisense)
5’- CCA GTC CAG AGT TCA GAA GAA AGG C -3’

Rat PRK S920A

DWS-78
5'- CCC ACA CTG GCC CCT CCC -3'

DWS-79
5'-GGC CTC CCC AGT GAA CTC -3'

Rat PRK D940A

DWS85
5'- G GAT TTC GCC TTT GTG GC -3'

DWS83
5'- CG GAA GGC CGC CTG CTC -3'

Rat PRK F939Y

DWS 437 (sense)
5’- C TTC CGG GAT TAC GAC TTT GTG G -3’

DWS 438 (Antisense)
5’- C CAC AAA GTC GTA ATC CCG GAA G -3’

Rat PRK F941A

DWS 476 (sense)
5’- CGG GAT TTC GAC GCA GTG GCA GGA GGC -3’

DWS 477 (antisense)
5’- GCC TCC TGC CAC TGC GTC GAA ATC CCG -3’

Rat PRK Δ940 F939A

DWS 474 (sense)
5’-G GCC TTC CGG GAT GCA TAA TTT GTG GC- 3’

DWS 475 (antisense)
5’-GC CAC AAA TTA TGC ATC CCG GAA GGC C -3’

Rat PRK Δ940 F939L
DWS 447 (sense)
5’- C TTC CGG GAT TTA TAA TTT GTG GCA GG -3’

DWS 448 (antisense)
5’- CC TGC CAC AAA TTA TAA ATC CCG GAA G -3’

Rat PRK Δ940 F939W

DWS 445 (sense)
5'-C TTC CGG GAT TGG TAA TTT GTG GCA G -3'

DWS 446 (antisense)
5'- C TGC CAC AAA TTA CCA ATC CCG GAA G -3'

Rat PRK Δ940 F939Y

DWS 443 (sense)
5'- C TTC CGG GAT TAC TAA TTT GTG GCA G -3'

DWS 444 (antisense)
5'- C TGC CAC AAA TTA GTA ATC CCG GAA G -3'

PRK ΔHR1

DWS 709 (sense)
5'-CTG CCT GCT GAT CAC CCC AAG -3'

DWS 710 (antisense)
5'- AGG TGC TGC CAG GTC TGC CCC AG -3'

Human PRK2

DWS 255 (sense)
5'- GCG TCC AAC CCC GAA AGA G -3'

DWS 305 (antisense)
5'- GCT GAT TGG TGT TAA GTC TAG AGA CAC -3'

Human PRK2 Δ979

DWS 872 (sense)
5'- GA GAT TTT GAC TAG ATT GCT GAT TGG TG -3'

DWS 873 (antisense)
5'- CA CCA ATC AGC AAT CTA GTC AAA ATC TC -3'

Human PRK2 Δ978

DWS 385 (sense)
5'- C AGA GAT TTT TAG TAC ATT GCT GAT TGG -3'

DWS 386 (antisense)
5'- CCA ATC AGC AAT GTA CTA AAA ATC TCT G -3'

Human PRK2 Δ977

DWS 516 (sense)
5'- G TTC AGA GAT TAG GAC TAC ATT GCT GAT TG -3'

DWS 517 (antisense)
5'- CA ATC AGC AAT GTA GTC CTA ATC TCT GAA C -3'

Human PRK2 Δ976

DWS 919 (sense)
5'- G TTC AGA TAG TTT GAC TAC ATT GCT GAT TGG -3'

DWS 920 (antisense)
5’- CCA ATC AGC AAT GTA GTC AAA CTA TCT GAA C -3’

Human PRK2 Δ974

DWS 383 (sense)
5’- GAG GAG CAG GAG ATG TAG AGA GAT TTT G -3’

DWS 384 (antisense)
5’- C AAA ATC TCT CTA CAT CTC CTG CTC CTC -3’

Human PRK2 Δ961

DWS 520 (sense)
5’- CTG ACT CCA CCA TGA GAA CCA AGG ATA C -3’

DWS 521 (antisense)
5’- G TAT CCT TGG TTC TCA TGG TGG AGT CAG -3’

Human PRK2 Δ960
DWS 522 (sense)
5’- CT ATT CTG ACT CCA TAG CGA GAA CCA AGG -3’

DWS 523 (antisense)
5’- CCT TGG TTC TCG CTA TGG AGT CAG AAT AG -3’

Human PRK2 Δ958

DWS 381 (sense)
5’- GCA CCT ATT CTG TAG CCA CCT CGA G -3’

DWS 382 (antisense)
5’- C TCG AGG TGG CTA CAG AAT AGG TGC -3’

Human PRK2 Δ950

DWS 379 (sense)
5’- GAT GAT GAA TAG ACC TCA GAA GCA C -3’

DWS 380 (antisense)
5’- G TGC TTC TGA GGT CTA TTC ATC ATC -3’

Human PRK2 Δ917

DWS 325 (sense)
5’- G CAC CCA TTT TTC TGA CTA ATT GAT TGG AGT GCT CTG ATG GAC -3’

DWS 330 (antisense)
5’- GTC CAT CAG AGC ACT CCA ATC AAT TAG TCA GAA AAA TGG GTG C -3’

Human PRK2 T958A

DWS 524 (sense)
5’- CA CCT ATT CTG GCT CCA CCT CGA G -3’

DWS 525 (antisense)
5’- C TCG AGG TGG AGC CAG AAT AGG TG -3’

Human PRK2 D978A

DWS 526 (sense)
5'- G TTC AGA GAT TTT GCC TAC ATT GCT G -3'

DWS 527 (antisense)
5'- C AGC AAT GTA GGC AAA ATC TCT GAA C -3'

Human PRK2 F977A

DWS 528 (sense)
5'- GAA ATG TTC AGA GAT GCG GAC TAC ATT GCT G- 3'

DWS 529 (antisense)
5'- C AGC AAT GTA GTC CGC ATC TCT GAA CAT TTC -3'

Human PRK2 F977L

DWS 530 **(s**ense)
5'- G TTC AGA GAT CTT GAC TAC ATT GCT G -3'

DWS 531 **(**antisense)
5'- C AGC AAT GTA GTC AAG ATC TCT GAA C -3'

Human PRK2 F977W

DWS 534 (sense)
5'- G TTC AGA GAT TGG GAC TAC ATT GCT GAT TG -3'

DWS 535 (antisense)
5'- CA ATC AGC AAT GTA GTC CCA ATC TCT GAA C -3'

Human PRK2 F977Y

DWS 532 (sense)
5'- G TTC AGA GAT TAC GAC TAC ATT GCT GAT TG -3'

DWS 533 (antisense)
5'- CA ATC AGC AAT GTA GTC GTA ATC TCT GAA C -3'

Human PRK2 Y979A

DWS 536 **(**sense)
5'- C AGA GAT TTT GAC GCG ATT GCT GAT TGG TG -3'

DWS 537 **(**antisense)
5'- CA CCA ATC AGC AAT CGC GTC AAA ATC TCT G -3'

Human PRK2 Y979L

DWS 538 (sense)
5'- C AGA GAT TTT GAC CTC ATT GCT GAT TGG -3'

DWS 539 (antisense primer)
5'- CCA ATC AGC AAT GAG GTC AAA ATC TCT G -3'

Human PRK2 Y979W

DWS 540 (sense)
5'- C AGA GAT TTT GAC TGG ATT GCT GAT TGG TG -3'

DWS 541 (Antisense)
5’- CA CCA ATC AGC AAT CCA GTC AAA ATC TCT G -5’

Human PRK2 Y979F

DWS 542 (sense)
5’- GA GAT TTT GAC TTC ATT GCT GAT TGG TG -3’

DWS 543 (antisense)
5’- CA CCA ATC AGC AAT GAA GTC AAA ATC TC -3’

Human PRK2 Δ978 F977A

DWS 1215 (sense)
5’- G TTC AGA GAT GCG TAG TAC ATT GCT GAT TG -3’

DWS 1216 (antisense)
5’- CA ATC AGC AAT GTA CTA CGC ATC TCT GAA C -3’

Human PRK2 Δ978 F977L

DWS 1217 (sense)
5’- G TTC AGA GAT TTA TAG TAC ATT GCT G -3’

DWS 1218 (antisense)
5’- C AGC AAT GTA CTA TAA ATC TCT GAA C -3’

Human PRK2 Δ978 F977W

DWS 1219 (sense)
5’- G TTC AGA GAT TGG TAG TAC ATT GCT G -3’

DWS 1220 (Antisense)
5’- C AGC AAT GTA CTA CCA ATC TCT GAA C -3’

Human PRK2 Δ978 F977Y

DWS 1221 (sense)
5’- G TTC AGA GAT TAC TAG TAC ATT GCT G -3’

DWS 1222 (antisense)
5’- C AGC AAT GTA CTA GTA ATC TCT GAA C -3’

Appendix C: Sequecing primers

DWS 43 (sense)
5'- CGAAATTAATACGACTCACT -3'

DWS 68 (antisense)
5'- GGGGAGGGGCAAACAACAGATG -3'

DWS 249 (sense)
5'- GCTGTTAAGAAGAAATCCTG -3'

Chapter 3:

Role of the PRK1 V5 domain in kinase function

This work has been published in:

Lim, W.G., et al., *The last five amino acid residues at the C-terminus of PRK1/PKN is essential for full lipid responsiveness.* Cell Signal, 2005. **17**(9): p. 1084-97.

3.1 Results

3.1.1 Generation of PRK1 Deletion and Point Mutants

This goal of this study was to determine the importance of individual residues and the collective contribution of the region within V5 to the catalytic activity of PRK1. A full-length rat PRK1 cDNA was cloned into mammalian expression vector pcDNA3. As the study involved serial deletion of C-terminal residues, the DNA sequence encoding a Myc-tag (EQKLISEEDL) was incorporated at the N-terminal before the second amino acid residue of PRK1 to facilitate the identification and purification of the recombinant PRK1. Point mutation and serial deletion of the C terminus was performed. PKA terminates at Phe^{350}, corresponding to Phe^{939} in rat PRK1. To this end, residues after the Phe^{939} of PRK1 (which contains 946 residues) were deleted to determine the role of these seven residues in conferring catalytic competence of PRK1. Highly conserved residues at the turn motif, hydrophobic motif and residues flanking these motifs were also deleted or mutated to elucidate their importance in catalytic activity and regulation of PRK1. The schematic diagram of the PRK1 domain structure and all the PRK1 constructs used in this study is shown in Fig. 3.1.

All mutants containing deletions, point mutations and the combination thereof were generated using a polymerase chain reaction (PCR)-based mutagenesis protocol with a circular plasmid as a template as described in Materials and Methods. The deletion mutants were generated by introducing an in-frame stop codon at the designated positions, e.g. the PRK1-Δ946 mutant carried a stop codon at position 946, yielding PRK1-(1-945). **All mutants were verified by gene sequencing and the results are listed in Appendix D.**

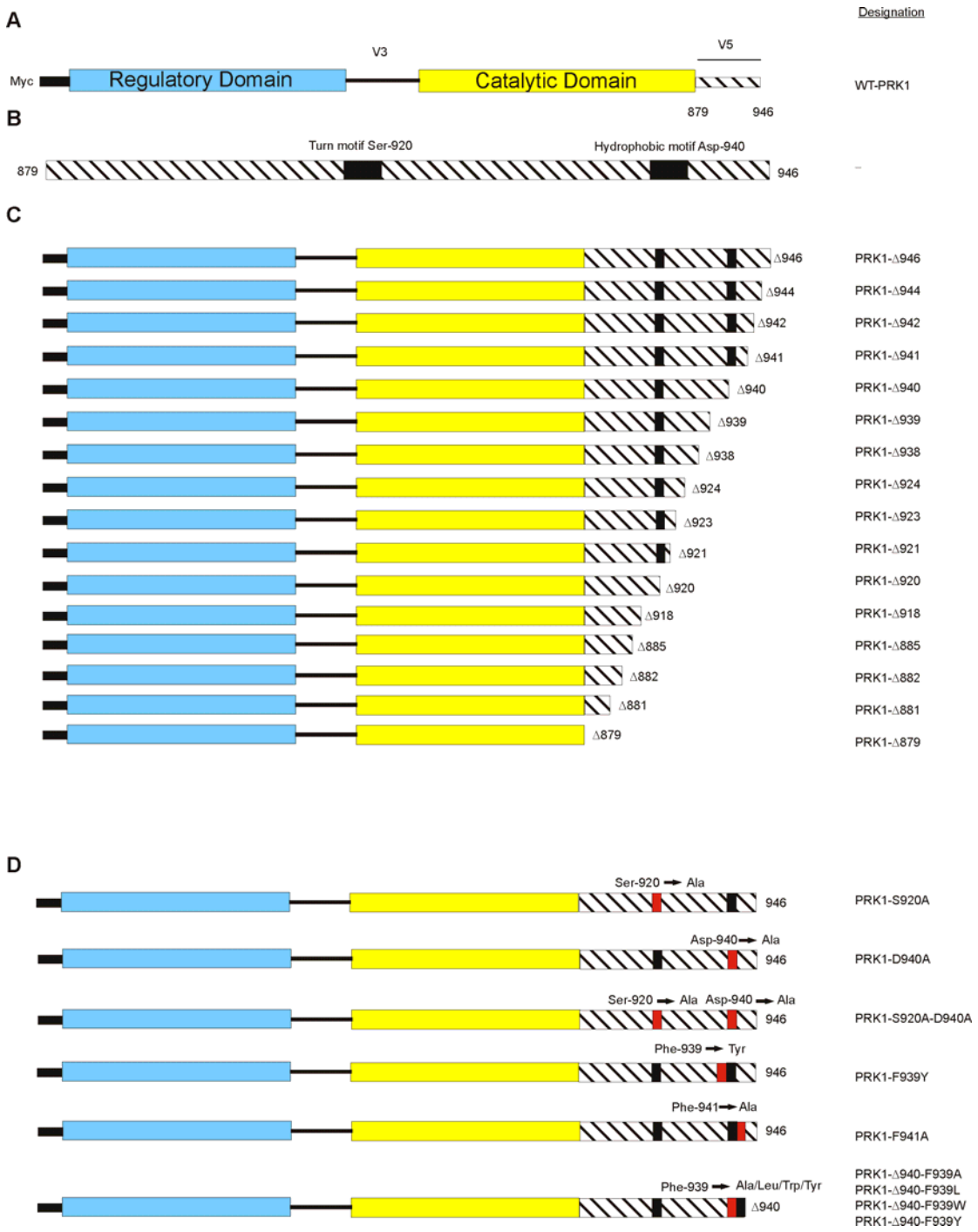


Fig. 3.1. Domain structure of PRK1 and PRK1 constructs used in this study. (A) Wild-type PRK1 (WT) consists of a regulatory domain (blue) and a catalytic domain (yellow), and the full length protein and mutants thereof were N-terminally tagged with a Myc epitope. The hinge region (V3), which links these two domains and the C-terminal segment (V5) are shown. Also shown are the turn motif (Ser-920) and the hydrophobic motif (Asp-940). **(B)** Enlarged view of the V5 domain showing the turn motif (Ser-920) and the hydrophobic motif (Asp-940). **(C).** Various PRK1 deletions were generated. The number at each C-terminus indicates the position of the stop codon, with the last expressed residue being the one just prior to the number. **(D)** Various point mutations generated in full length and deleted PRK1, where S920A indicate the mutation of serine to alanine at position 920. The whole PRK1 molecule and the enlarged V5 domain illustrated are not drawn to scale.

3.1.2 The Hydrophobic Motif is Not Required for the Solubility of PRK1

PKA terminates at Phe^{350}, corresponding to Phe^{939} in rat PRK1. The phosphor-acceptor at the hydrophobic motif in rat PRK1 is a phosphomimetic which corresponds to the residue Asp^{940}. In PKCα [95] and PKCβII [96], mutation of the phosphorylatable residue of the activation loop to a neutral, nonphosphorylatable residue resulted in the expression of a dephosphorylated protein that accumulates in the detergent-insoluble fraction. It has been proposed that PKC mutants lacking the C-terminal segment might be insoluble [45], thus it was important to analyse the solubility of the C-terminal deletion mutants of PRK1. The requirement of the hydrophobic phosphorylation motif in the solubility of PRK1 was explored by generating a PRK1 deletion mutant (PRK1-Δ940) that lacks the segment spanning the phosphorylation mimetic Asp^{940} and the C-terminal residue.

COS cells were used to ensure proper folding and post-translational modifications of the recombinant PRK. Furthermore, COS cells have a SV40 T antigen which will bind to the SV40 origin of replication sequence in the pcDNA3 vector, that will cause the amplification of the vector so that one obtains higher yield than other cell lines that do not have SV40 T antigen.

When introduced into COS-1 cells by transient transfection, there was little appreciable change in the distribution of ectopically expressed PRK1-Δ940 in the supernatant and pellet fractions upon cell lysis by Triton X-100 compared to that of the wild-type PRK1 (Fig. 3.2). Furthermore, other mutants in this region, PRK1-Δ940+F939Y, PRK1-F939Y, PRK1-D940A and PRK1-F941A, also displayed no

significant change in the solubility of the mutant PRK1 as shown in Fig. 3.2. In addition, mutants in which the phosphor-acceptor and the negative charge at the turn motif and hydrophobic motif (PRK1-S920A+D940A) were eliminated also showed no discernible changes in the solubility of the mutant PRK1. Surprisingly PRK1-Δ939 exhibited significantly reduced solubility compared to PRK1-Δ940 mutant (Fig. 3.2). Deletion at the turn motif phosphorylation site (Ser^{920}) resulted in a further decrease in the solubility of the mutant PRK1 (PRK1-Δ920 and PRK1-Δ918). Removal of the entire V5 domain (PRK1-Δ879) resulted in a mostly insoluble protein (Fig. 3.2). Thus, the negative charge at the hydrophobic motif and the phosphorylation of the turn motif did not contribute significantly to the solubility of PRK1. Rather, the physical presence of Phe^{939}, which is absolutely conserved in AGC kinases, and the integrity of the N-terminal portion of the V5 domain (residues 878 to 920) played critical roles in conferring detergent solubility and subcellular localization to PRK1.

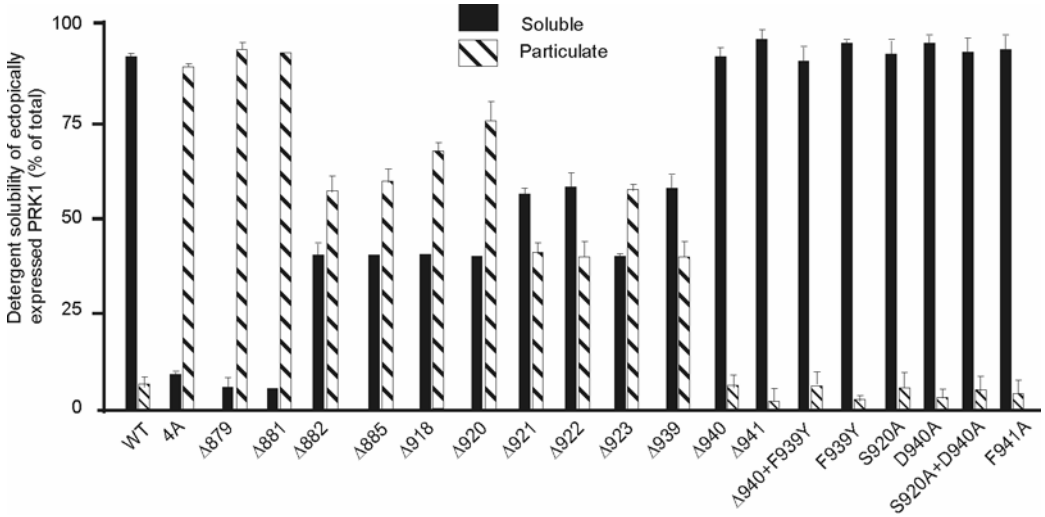


Fig. 3.2. **Determinants of detergent solubility of PRK1 in the C-terminus of the catalytic domain.** Expression constructs for Myc-tagged wild-type PRK1 and PRK1 point mutants and/or deletion mutants were transiently transfected into COS-1 cells. Twenty hours post-transfection, cells were lysed with ice-cold buffer A containing 1% Triton X-100 and the detergent-soluble supernatant and detergent–insoluble particulate fractions were obtained after centrifugation. Approximately 18% of the supernatant and all of the particulate fractions were subjected to SDS-PAGE and Western blotting using anti-Myc (9E10). The construct 4A is a Myc-PRK1 mutant with all the four potential phosphor-acceptor serine/threonine residues in the activation loop changed to alanine.

The percentage of PRK1 in detergent-soluble (Soluble) and detergent-insoluble (Particulate) fractions in each sample is shown. Data presented as means ± s.e.m.; n=4.

3.1.3 The Highly Conserved Phe^{939} but Not the Phosphorylation Mimetic Asp^{940} Is Absolutely Required for the Catalytic Competence of PRK1

Previous structural studies linked phosphorylation of the hydrophobic motif to the stabilization of the kinase core, where mutation of the conserved serine or threonine residue at the hydrophobic motif to an alanine residue in PKC revealed that phosphorylation of the hydrophobic motif was not required for PKC function; rather, it stabilized the enzyme [129, 130, 330, 331]. However, it is noteworthy that in those studies, the contribution of the hydrophobic motif to kinase activity was determined in isolation via point mutation at a single residue. Point mutation studies are useful to elucidate the importance of single residues; however, such studies do not probe the combined interactions of adjacent residues. Thus, my study adopted a more comprehensive approach to determine the role of the C-terminus in kinase activity. Point mutations were generated to determine the importance of each residue; concurrently, C-terminal residues were serially deleted to assess the collective contributions of the C-terminal residues to PRK1 catalytic activity. Autophosphorylation assay was chosen to assess PRK1 kinase activity because such analysis examines the ability of a kinase to phosphorylate its true physiological substrate and therefore is a better indication of the intrinsic catalytic activity. As a control, the kinase-inactive quadruple mutant of PRK1 (i.e. T776A, T777A, T778A and T782A), termed 4A was generated in which all phosphorylatable threonine residues in the activation loop in PRK1 (T778) were mutated to alanine in order to prevent compensating phosphorylation.

Fig. 3.3*A* and 3.3*B* show that the removal of the C-terminal tail up to Phe^{941} in PRK1, or even inclusive of the phosphomimetic Asp^{940} (i.e. PRK1-Δ942, PRK1-Δ941 and PRK1-Δ940) had little impact on catalytic competence. However, the catalytic activity diminished by 95% once Phe^{939} was deleted (i.e. PRK1-Δ939). To assess whether the loss of kinase activity was due to absence of residues 940-946 or the absence of Phe^{939}, phenylalanine at position 939 was replaced with various other residues in the background of PRK1-Δ940 mutant, in which the last amino acid residue is Phe^{939} and hence a mimetic of the C-terminus of PKA. Notably, catalytic activity was lost with either conservative substitution of Phe^{939} (tyrosine or tryptophan) or substitution with non-polar residues with similar hydropathy index (alanine or leucine) (Fig. 3.3C, 3.3D). Identical results were obtained in transphosphorylation assays employing a peptide derived from ribosomal protein S6 peptide as a substrate for all the PRK1 mutants shown in Fig. 3.3. Therefore, it appears that the C-terminal extension residues 940-946 of PRK1 in the V5 domain, which are absent in PKA, are dispensable for the catalytic competence of PRK1. Once the C-terminal extension is removed, the side chain of Phe^{939} is absolutely necessary for activity, most likely by keeping the rest of the catalytic domain of PRK1 in an active conformation. Apparently, the structural function performed by the phenyl ring of Phe^{939} in PRK1-Δ940 mutant cannot be mimicked by the phenyl ring of tyrosine or tryptophan, consistent with the fact that phenylalanine is an invariant residue at this position all AGC [112].

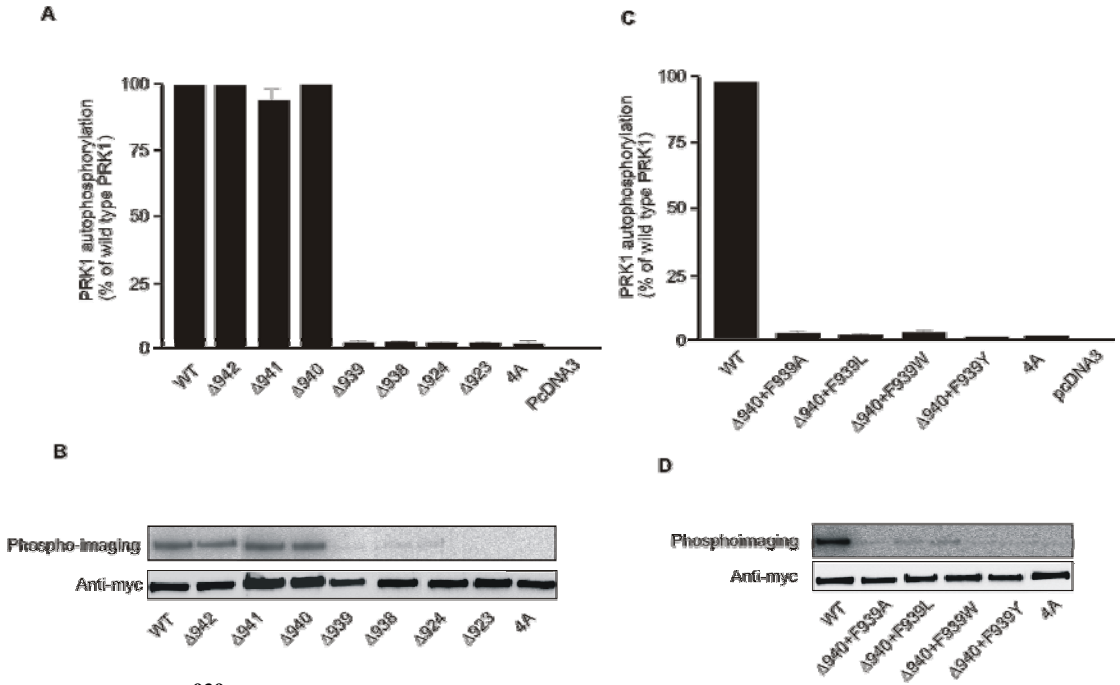


Fig. 3.3. **Phe^{939} but not the C-terminal extension in the V5 domain of PRK1 is required for the catalytic competence of the kinase**. Myc-tagged PRK1 expression constructs were transiently transfected into COS-1 cells. At 20 h post-transfection, cells were lysed and the exogenous wild-type or mutant PRK1 were immunoprecipitated with anti-Myc antibodies. The catalytic activity of PRK1 was determined in an autophosphorylation assay and is expressed as percentage of the activity of wild-type PRK1. All reactions proceeded linearly with time. 3*A*, 3*C*, the autophosphorylation activities of PRK1 mutants are compared with that of wild-type kinase (means ± s.e.m.; n=3). 3*B*, 3*D*, representative autoradiographs and corresponding Western blots used to determine the apparent molecular mass of the mutant protein encoded by each construct and to derive the specific activity of PRK1. The protein 4A, a Myc-PRK1 mutant in which all four potential phosphor-acceptor serine/threonine residues in the activation loop were changed to alanine.

3.1.4 A Network of Intramolecular Interactions in the V5 Domain Contributes to the Catalytic Competence of PRK1

To compare the relative importance of Phe^{939} and the last seven amino acids in the V5 domain to catalytic competence in PRK1, Phe^{939} was mutated in the full length PRK1 construct. In the full-length PRK1, two phenylalanines, Phe^{939} and Phe^{941}, surround the phosphorylation mimetic Asp^{940}. The Phe^{939} is highly conserved among the PKC members, whereas Phe^{941} is less conserved and may be replaced with a tyrosine residue in PRK2 and PKCα. To determine the relative importance between the two conserved hydrophobic residues for catalytic competence, Phe^{941} was mutated to aromatic residues (tyrosine or tryptophan) or non-polar residues with similar hydropathy index (alanine or leucine) in a full length PRK1 and compared with similarly mutated Phe^{939} mutants. The effect of these mutations in the conserved residues in the V5 domain of PRK1 on the catalytic activity of full-length PRK1 was analysed in a kinase assay.

Mutations at Phe^{939} were not tolerated whereas mutations at Phe^{941} had much less deleterious impact on the catalytic competence of the mutant enzyme (Fig. 3.4*A, B*). Intriguingly, the kinase activities of these Phe^{939} point mutants are in sharp contrast with the Phe^{939} deletion mutant in which resides 940 to 946 were removed, resulting in a deleted PRK1 that was almost as active as the wild-type enzyme (Fig. 3.3*A*). The activity of Phe^{939} mutants varied from 50% (F939W) to less than 10% (F939A, F939L and F939Y). On the other hand, the activity of the Phe^{941} mutants F941W, F941L and F941Y was >50%, although the mutant F941A had greatly reduced activity (25%).

The phosphor-acceptor or phosphomimetic residues in the turn motif and hydrophobic motif (Ser^{920} and Asp^{940}) were mutated to alanine to determine the importance of these conserved residues. In line with previous studies where mutation of the phosphor-acceptor impaired catalysis significantly [103, 104], the mutant PRK1-S920A had negligible catalytic activity (Fig. 3.4A). However, the finding that PRK1-D940A was also catalytically inactive (Fig. 3.4A) was unexpected because the PRK1-Δ940 deletion mutant, lacking Asp^{940} was fully active (Fig. 3.3*A*). Moreover, mutation of Phe^{939} to tryptophan in PRK1-Δ940 deletion (PRK1-Δ940+F939W, Fig. 3.3*C*) resulted in an inactive kinase, but the same mutation introduced to the full-length PRK1 (PRK1-F939W) yielded at least 55% of the catalytic activity compared with that of the wild-type PRK1 (Fig. 3.4*A).*

These seemingly contradictory and unexpected results suggest that residues within the V5 domain interact with each other and with those in the catalytic core. The overall impact on the establishment of a catalytically competent conformation of the catalytic core must be governed by the outcome of a network of intramolecular interactions that depend on the context of the C-terminal extension Phe^{941} in PRK1.

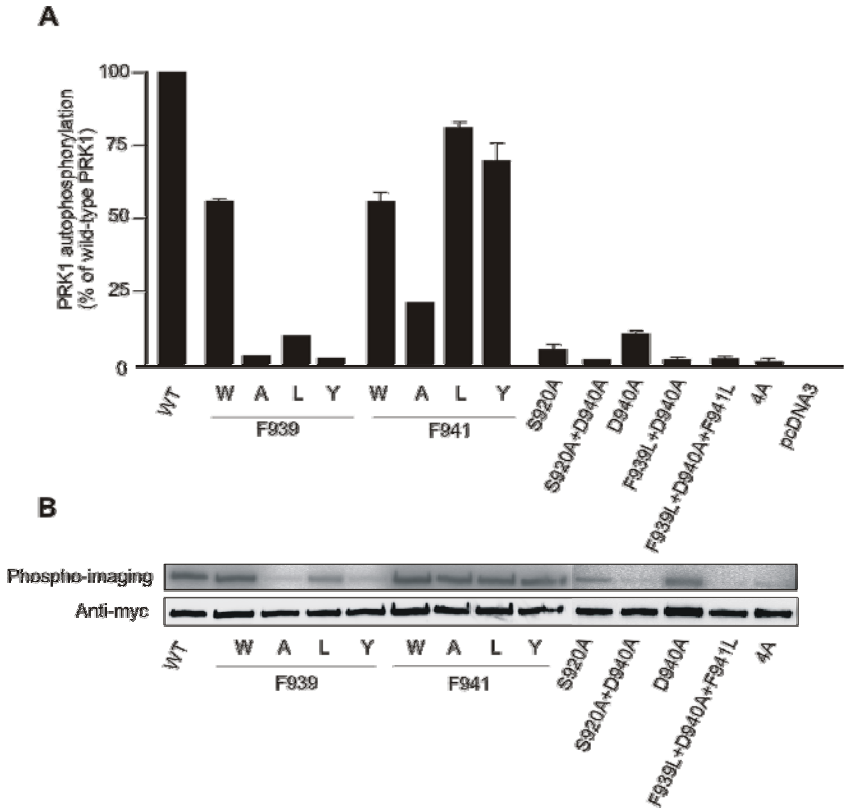


Fig. 3.4. **Contributions of several key amino acid residues in turn motif and hydrophobic motif to the catalytic competence of PRK1**. Plasmids encoding Myc-tagged wild-type and mutant PRK1 were introduced into COS-1 cells. Twenty hours post-transfection, cells were lysed and ectopically expressed PRK1 was immunoprecipitated with anti-Myc antibodies. The specific activity for autophosphorylation of PRK1 was analyzed in an immune-complex kinase assay *in vitro*. The means ± s.e.m of autophosphorylation (percentage of that of wild-type PRK1) of three independent experiments are shown (4*A*), along with a typical autoradiograph and a Western blot used to derive the specific activity (4*B*). 4A, a Myc-PRK1 mutant with all the four potential phosphor-acceptor serine/threonine residues in the activation loop changed to alanine.

3.1.5 The C-terminal Tail Of PRK1 Is Critical in Conveying Stability to the Kinase *in vivo*

Despite a single residue difference, there exists distinct divergence in catalytic activity between PRK1-Δ940 and PRK1-Δ939. A relevant question at this juncture would be whether the deletion of the C-terminal residues results in PRK1 adopting a structural conformation that is more susceptible to degradation. Consequently, PRK1-Δ939 had reduced activity because of decreased structural stability. Thus, the stability of PRK1 V5 domain deletion mutants was assessed by determination of the heat stability

replicating *in vitro* kinase assay conditions. While *in vitro* kinase assay was performed at 30 °C for 10 min, the *in vitro* heat stability was carried out at 30 °C for an extended period of 30 min to study the effect of heat on PRK1 stability. Deletion that removes the C-terminal tail that is absent in PKA had no gross negative effect on the *in vitro* heat stability of the PRK1-Δ940 mutant as it exhibited similar activity before and after heat treatment (Fig. 3.5*A*). From Fig. 3.5*B*, it is apparent that there was little degradation of PRK1 deletion mutants after incubation at 30 °C for 30 min *in vitro*. Thus, the negligible kinase activity of PRK1-Δ939 observed (Fig. 3.3A) was most likely a result of impaired catalysis rather than the degradation of the mutant.

The heat stability assay was indicative of the stability of the deletion mutants under *in vitro* conditions. To reflect the physiological stability, the half-lives of PRK1 mutants ectopically expressed in COS-1 cells were determined under *in vivo* condition. Cycloheximide is a glutarimide antibiotic that inhibits protein synthesis in systems that utilize ribosomes of the 80S type [332]. Cycloheximide exerts its effect by interfering with peptidyl transferase activity of the 60S ribosome, thus blocking translational elongation. COS-1 cells transiently transfected with PRK1 expression constructs were incubated with 200 μg/ml cycloheximide for several hours as this treatment had been shown to effectively inhibit protein synthesis in this cell line [333, 334]. The transfected cells were incubated with cycloheximide for 4 h to ensure complete inhibition of protein synthesis before the first lysis which was designated as time zero, with subsequent cell lysis performed every 2 h. Thirty percent of the total cell lysate from the different time points was loaded in SDS-PAGE and analysed with anti-Myc antibody in Western blotting to determine the relative ectopically expressed PRK1. The amount of actin for each lane was determined with actin specific antibody

to normalize differences in the amount of total cellular proteins in each lane. During the experiments, cycloheximide was replenished every 8 h to ensure near-complete inhibition of protein synthesis of COS-1 cells.

The half-lives of total cellular PRK1 and soluble PRK1 were measured separately, as the former represents the half-life of both detergent-soluble and detergent-insoluble pools of PRK1 while the latter represents the half-life of the detergent-soluble (mainly cytosolic) pool of PRK1. Data presented in Table 3.1 indicate that for WT-PRK1, the turnover of the detergent-insoluble fraction of PRK1 was faster than that of the detergent-soluble fraction. Removal of the segment of Phe^{941}-Tyr^{946} resulted in an approximately nine-fold reduction in the stability of the mutant PRK1 in both the total cellular pool and the detergent-soluble (cytosolic) pool *in vivo*. Further deletion of the V5 domain of PRK1, including the removal of the entire V5 segment (the PRK1-Δ879 mutant) did not shorten the half life of PRK1 any further. Thus, the C-terminal segment (residues 941-946) of the V5 domain of PRK1 that does not exist in PKA conferred the stability to the kinase against degradation by intracellular protein destruction machineries.

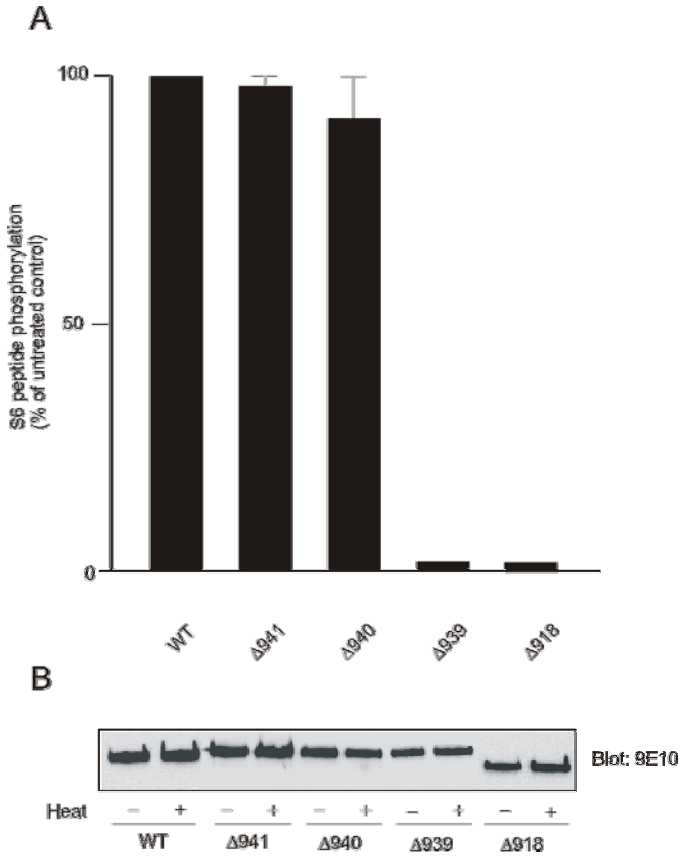


Fig. 3.5. **Effect of the removal of the C-terminal extension of V5 domain in PRK1 has little impact on heat stability of the kinase**. Myc-tagged PRK1 expression constructs were introduced into COS-1 cells. Twenty hours post-transfection, exogenously expressly PRK1 was immunoprecipitated. After three stringent washes, the immune-complexes were incubated at 30 °C for 30 min before proceeding to a peptide phosphorylation assay. The specific activity of each PRK1 after heat treatment is expressed as percentage of the activity of the untreated control sample and is shown as means ± s.e.m of three independent experiments (Fig. 3.5*A*). A representative Western blot showing the mass of the proteins before and after heat treatment is shown in Fig. 2.5*B*.

TABLE 3.1

Apparent half-life of exogenous PRK1 in COS-1 cells

PRK1	WT PRK1	Δ879	Δ939	Δ940	Δ941	Δ942	Δ943
Soluble (h)	64 ± 5	6 ± 0.3	6 ± 0.1	6 ± 0.1	6 ± 0.4	6 ± 0.4	6 ± 0.1
Total (h)	51± 3.0	5 ± 1.0	5.75 ± 0.25	5.5 ± 0.5	6.5 ± 1.5	6.5 ± 1.5	6 ± 1.5
Comparison between soluble and total half-life	p<0.05	p>0.05					

3.1.6 The Full-Length Hydrophobic Motif Is Dispensable for the Phosphorylation of the Activation Loop of PRK1 by PDK-1

It has been proposed that the hydrophobic motif of PKC isozymes is the anchor for docking with PDK-1 [270]. The phosphorylation of a conserved threonine residue at the activation loop in isozymes of the PKC superfamily by PDK-1 is the first and rate-limiting step in the maturation process of this group of serine/threonine kinases [45].

Fig. 3.3A shows that in the absence of the hydrophobic motif (Asp^{940}) and its C-terminal residues, PRK1-Δ940 mutant displayed kinase activity as high as that of the wild-type PRK1. Yet, PRK1 Phe^{939} point mutants with complete hydrophobic motif and C-terminal residues had significantly decreased kinase activity. While the extent of activation loop phosphorylation is not proportional to kinase activity, it may serve as an indicator of the catalytic competence of the kinase. Thus the phosphorylation status of the activation loop of the PRK1 Phe^{939} point mutants and PRK1 deletion mutants was investigated to determine the relationship between activation loop phosphorylation and catalytic competence and the requirement for the C-terminal residues for activation loop phosphorylation.

To determine if Asp^{940} which corresponds to the phosphorylation mimetic residue of the hydrophobic motif is required for activation loop phosphorylation, PRK1-Δ940 was expressed in COS cells and deleted proteins were immunoprecipitated followed by Western blot analysis using the P500 antibody that specifically recognizes the phosphorylated activation loop of PKCs [97, 335]. As has been demonstrated previously by others [97, 335], the P500 antibody is highly specific for phosphorylated activation loop as it did not react with the PRK1-4A mutant in which

all the four possible phosphor-acceptor serine/threonine residues in the activation loop are replaced with alanine residues (Fig. 3.6 top panel). PRK1-Δ938, PRK1-Δ939 and PRK1-Δ941 were also examined for the phosphorylation of the activation loop to test for correlation with earlier data (Fig. 3.3B). In contrast to what has been reported for PRK2 [270], the deletion of either the segment of Phe^{941}-Tyr^{946} (PRK1-Δ941) or that of Asp^{940}-Tyr^{946} (PRK1-Δ940) had no significant effect on the extent of the phosphorylation of the activation loop of PRK1 (Fig. 3.6, top panel). Deletion of the segment of Phe^{939}-Tyr^{946} (PRK1-Δ939) or that of Asp^{938}-Tyr^{946} (PRK1-Δ938) of PRK1 abolished the phosphorylation at the activation loop. Thus, it appears that phosphorylation mimetic residue of Asp^{940} and the succeeding hydrophobic residue of Phe^{941} are not required for activation loop phosphorylation, since the absence of both (as evident in PRK1-Δ940) still resulted in an activation loop phosphorylated PRK1. This finding corresponds to the kinase activity of the respective PRK1 deletion mutants: PRK1-Δ941 and PRK1-Δ940 were phosphorylated at the activation loop and had kinase activity as high as that of the wild-type PRK1 while PRK1-Δ939 and PRK1-Δ938 lacked activation loop phosphorylation and were catalytically inactive (Fig. 3.3A).

To study further whether phenylalanine is absolutely required at position 939 for the phosphorylation of the activation loop, a series of different point mutations at position 939 in the full-length protein was introduced. As shown in Fig. 3.6, the PRK1-F939Y mutant had a slightly reduced phosphorylation at its activation loop. In contrast, the PRK1-F939W mutant had a similar, if not higher, extent of phosphorylation at its activation loop. The idea that phenylalanine at position 939 is not absolutely required for the phosphorylation at the activation loop was further strengthened by the finding

that PRK1-F939L or even F939A mutants had reduced but still readily detectable phosphorylation at the activation loop (Fig. 3.6). The phosphorylation status of these PRK1 point mutants paralleled the kinase activity where PRK1-F939A exhibited low activation loop phosphorylation and low kinase activity, and PRK1-F939W had a higher level of activation loop phosphorylation and a corresponding higher level of kinase activity. It should be noted that while there is a relation between activation loop phosphorylation and kinase activity, the two parameters are not directly proportional, i.e. a higher level of activation loop phosphorylation does not necessarily equate to a higher level of kinase activity.

Thus, the phosphorylation mimetic residue of Asp^{940} and the hydrophobic residue of Phe^{941} are not required for activation loop phosphorylation, as demonstrated by PRK1-Δ940; the absence of both residues still resulted in an activation loop phosphorylated PRK1. However, the absence of another hydrophobic residue at position 939 (PRK1-Δ939) resulted in a PRK1 mutant that was not phosphorylated at the activation loop. Taken together, these results provide evidence that the full length hydrophobic motif in PRK1 is not necessary for the activation loop phosphorylation by PDK-1.

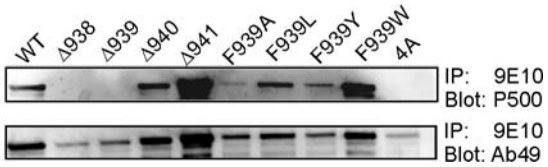


Fig. 3.6. Phosphorylation of consensus PDK-1 phosphorylation motif in the activation loop of PRK1 deletion mutants. Twenty hours post-transfection, myc-tagged PRK1 mutants were immunoprecipitated from COS-1 cells and subjected to Western blot analysis using P500 antibody followed by stripping and reprobing with anti-PRK1 regulatory domain antibody (Ab49). Data shown are typical of 6 independent experiments. 4A, a Myc-PRK1 mutant with all the four potential phosphor-acceptor serine/threonine residues in the activation loop changed to alanine. IP: immunoprecipitation

3.1.7 Interaction of PDK-1 with PRK1 and the Productive Phosphorylation of the Activation Loop Are Separate Biochemical Events

The docking of PDK-1 to the hydrophobic motif of PKC was proposed to be central to the regulation of PKC activity. The current working model of PDK-1 interaction with the AGC family of serine/threonine protein kinases states that PDK-1 binds with the hydrophobic motif of its substrate kinases (such as PKCs) and subsequently phosphorylates its substrate's activation loop [270]. In PRK2, mutation of the conserved aromatic residues in the hydrophobic motif was reported to result in the inhibition of the interaction of PRK2 with PDK-1 [108]. Data presented in Fig. 3.6 indicate that in PRK1, the mutation of conserved phenylalanine residues at the hydrophobic motif (PRK1-F939A, PRK1-F939L, PRK1-F939W and PRK1-F939Y) does not prevent PDK-1 interaction. In this section, the question of whether the hydrophobic motif, the entire V5 domain or even the activation loop itself of PRK1 is required for the physical interaction between PDK-1 and PRK1 *in vivo* is addressed.

The *in vivo* binding of PDK-1 to PRK1 was analysed in a co-transfection and co-immunoprecipitation assay in which PRK1 deletion mutants and PDK-1 were coexpressed in COS cells. After cell lysis, PRK1 mutants were immunoprecipitated and the immunoprecipitates were washed twice sequentially with 1% NP-40 and 300 mM NaCl to remove non-specific binding. The purified immunoprecipitate was subsequently analysed in SDS-PAGE and Western blot with antibody against PDK-1 to detect PDK-1 interaction with PRK1 (Fig. 3.7A, top panel). As PDK-1 and PRK1 deletion mutants have similar molecular mass, the blot was stripped off PDK-1-specific antibody and reprobed with antibody against PRK1 to determine the amount of PRK immunoprecipitated (Fig. 7B, 2nd panel). To determine the expression level of

PDK-1, 20% of cell lysate was loaded in a separate SDS-PAGE and analysed (Fig. 3.7A, 3rd panel). The amount of beads used for each immunoprecipitation was analysed by bands corresponding to IgG heavy chain bands (Fig. 7A, last panel). The specificity of the co-immunoprecipitation was confirmed in control experiments in which a Myc-tagged PRK1 regulatory domain-only construct failed to immunoprecipitate the co-transfected Myc-PDK-1 (Fig. 3.7*C*, *middle panel*).

As shown in Fig. 3.7, the PRK1-Δ918 mutant in which the C-terminal segment containing turn motif was removed could still bind with PDK-1, indicating that the hydrophobic motif of PRK1 is dispensable for the physical interaction with PDK-1. Surprisingly, PRK1 mutants with further deletions into the catalytic domain all retained the capacity of interaction with PDK-1 (Fig. 3.7*A*). These mutants include the PRK1-Δ860 mutant in which the entire V5 segment was removed, the PRK1-Δ830 and PRK1-Δ800 mutants in which part of the catalytic core was removed and even the PRK1-Δ770 mutant in which the activation loop was deleted. The binding of PDK-1 to the above PRK1 mutants was confirmed in the reciprocal immunoprecipitation (Fig. 3.7*B*). The data in Fig. 3.7*A*-*C* also suggest that PDK-1 could interact with PRK1 in the segment between the hinge (V3) region and NH_2 terminus of the activation loop of PRK1 *in vivo*, at least under the experimental conditions of this study.

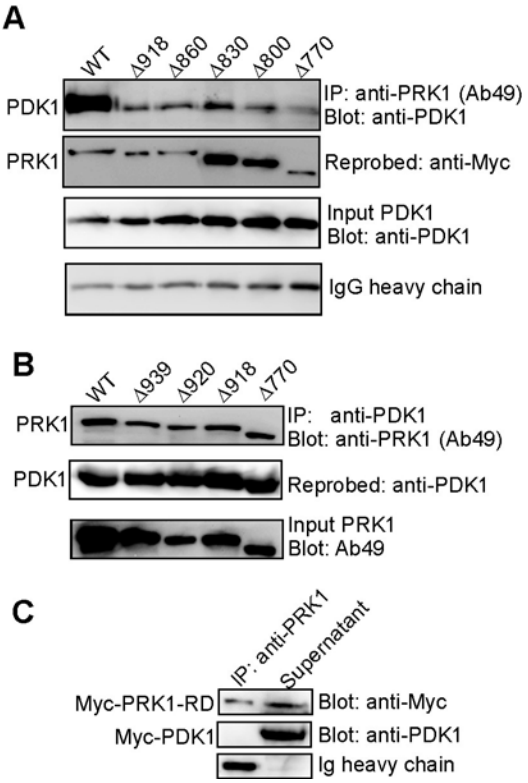


Fig. 3.7. Co-immunoprecipitation of PDK-1 with various PRK1 deletion mutants. COS-1 cells were transiently co-transfected with Myc-PDK-1 and one of the Myc-PRK1 plasmids as indicated. Twenty hours post-transfection, cells were lysed. *A*, PRK1 were immunoprecipitated using an anti-PRK1 regulatory domain antibody (Ab49), and co-immunoprecipitation of PDK-1 was evaluated by Western blot analysis with an anti-PDK-1 antibody. The blot was stripped and re-probed with anti-Myc. Approximate 20% of the total input PDK-1 was evaluated with Western blot analysis. *B*, Co-immunoprecipitation of PRK1 with PDK-1 was evaluated by Western blot analysis with anti-PRK1 antibody (Ab49). Twenty percent of input PRK1 was evaluated in Western blot using Ab49. *C*, Myc-PDK-1 was co-transfected with Myc-PRK1 regulatory domain-only construct. The possible interaction between PDK-1 and PRK1 regulatory domain was evaluated by immunoprecipitation with anti-PRK1 (Ab49) followed by Western blot analysis with anti-Myc. Results are typical of three independent experiments.

3.1.8 The C-terminal Portion of the V5 Domain of PRK1 Is Critical for Full Lipid Responsiveness

PRK1 is activated by a variety of lipids, among which arachidonic acid is one of the best characterized lipid activators [53]. The arachidonic acid binding site in PRK1 has been mapped to the C-terminal portion of the regulatory domain of PRK1 (residues 455-511) [267]. It is noteworthy that Ono and colleagues [267] mapped the

arachidonic acid binding site in PRK1 by systematically truncating the N-terminal half of PRK1. While PRK1 N-terminal deletion mutant 455-946 was found to have low basal activity similar to the wild type PRK1, and was highly dependent on arachidonic acid, the PRK1 N-terminal deletion mutant 511-946 had higher basal activity with only a slight dependence on arachidonic acid. Thus a region N-terminal to the hinge region (residues 455-511) was mapped as the region that binds arachidonic acid. However, the precise mechanism by which this region regulates the catalytic domain of PRK1 has not been elucidated.

The present study demonstrated that the V5 domain is critical for catalytic function because the removal of seven residues resulted in an inactive kinase (Fig. 3.3A). A possibility for the lack of activity in PRK1-Δ939 is that the last seven residues are necessary for maintaining the catalytic domain in an optimal conformation for catalysis, absence of which results in PRK1 inability to phosphorylate its substrate. To address this possibility, PRK1 C-terminal residues were deleted sequentially and tested for catalytic activity with and without lipid activator. To this end, PRK1 deletion mutants were over-expressed in COS cells and immunoprecipitated. Immunoprecipitated PRK1 mutants were equally divided into two tubes to ensure that similar amount of proteins were used. Subsequently, PRK1 deletion mutants were tested for their control of lipid responsiveness by analysing the ability to transphosphorylate a S6 peptide substrate in the presence or absence of arachidonic acid.

As shown in Fig. 3.5*A* and Fig. 3.8, the deletion of amino acid residues between 940 to 946 did not have any gross detrimental effect on the basal catalytic activity of

PRK1. However, removal of the last three amino acid residues from the C-terminus of PRK1 (the PRK1-Δ944 mutant) resulted in reduced activation by arachidonic acid (Fig. 3.8). Intriguingly, further deletion of the C-terminal portion of the V5 domain of PRK1 (PRK1-Δ942, Δ941 and Δ940 mutants) almost abolished the lipid responsiveness of PRK1 to arachidonic acid (with only 0.2-0.25-fold activation). Deletion at Phe^{939} resulted in a PRK1-Δ939 mutant that not only lost its basal catalytic activity but also could not be revived/activated by arachidonic acid, indicating that the last seven amino acids in the V5 domain are necessary for maintaining an optimal conformation of the catalytic domain. Thus, a novel element that controls the lipid responsiveness of PRK1 at the extreme C-terminus of the V5 domain was serendipitously identified.

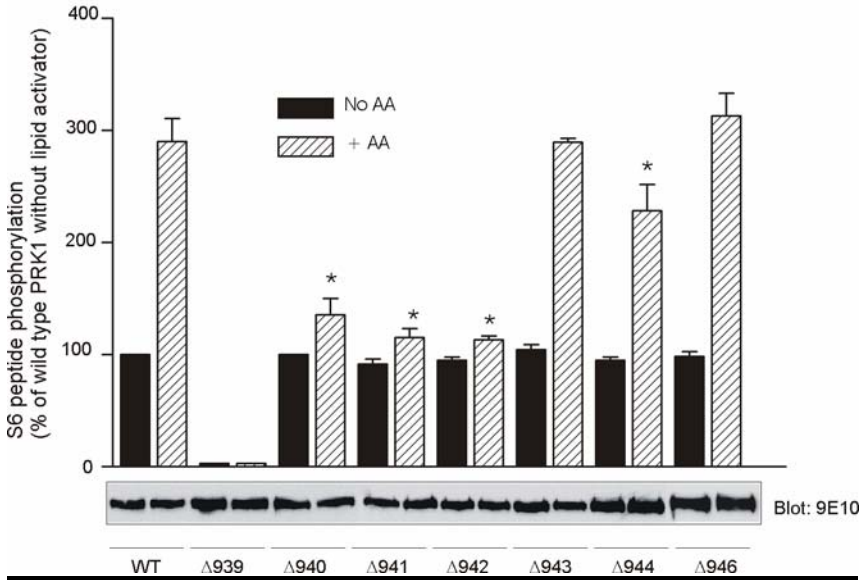


Fig. 3.8. ***In vitro*** **arachidonic acid responsiveness of wild-type PRK1 and PRK1 deletion mutants**. Myc-tagged PRK1 expression constructs were transiently transfected into COS-1 cells. Twenty hours post-transfection, PRK1 was immunoprecipitated with anti-Myc (9E10) antibody. The catalytic activity of various PRK1 constructs towards an S6 peptide was analyzed in an immune-complex kinase assay with or without 40 μM arachidonic acid solublised in 20mM Tris, pH 7.5. Data are expressed as percentage of activity of wild-type PRK1 and are means ± s.e.m. of three independent experiments. A representative Western blot used to derive the

specific activity of PRK1 is shown at the bottom. AA, arachidonic acid. *, $p<0.01$ using single-factor ANOVA.

3.1.9 Computer Modeling of Three-Dimensional Structure of the Catalytic Domain of PRK1

Sequence alignment is a central technique in homology modeling used to establish the correspondence of each amino acid between the reference protein(s) and those of the unknown proteins, providing a basis for transferring coordinates from the reference to the model protein. The overall folding topology and patterns of all mammalian AGC family of serine/threonine kinases are very similar [85, 107]. Therefore, homology modeling based on protein sequence alignment may be useful in predicting the three-dimensional structure of the catalytic domain of PRK1. The alignment of the amino acid sequence of the catalytic domain of rat PRK1 with that of human PKBβ and the catalytic subunit of mouse PKA guided by the crystal structures of the latter kinases [85, 336] is shown in Fig. 3.9*A*. Rat PRK1 has 39% and 43% sequence identity with mouse PKA (1APM) and human PKBβ (1O6L), respectively. Notably, the amino acid sequence of PKA terminates at Phe^{350} (corresponding to Phe^{939} in rat PRK1), while PKBβ [337] terminates at Glu^{481} (Arg^{480} in human PKBβ corresponds to Tyr^{946} in rat PRK1). To gain further insights into the conformation and properties of the catalytic domain of PRK1, in particular the role of the V5 segment in the establishment of the catalytic competence of the kinase, a three-dimensional structure model of the catalytic domain of PRK1 was built (Fig 3.9 *B-D*) based on the X-ray structures of PKA and PKB that contain the structures of the respective V5 domain [85, 336].

The three-dimensional model of the catalytic domain of rat PRK1 (residues 612 to 946, including the C-terminus of the kinase) consists of an N-lobe and a C-lobe linked

by the V5 segment (Fig. 3.9*A*, *B*), consistent with conserved packing as shown for PKA and PKB. The absolutely conserved ATP-binding motif (Asp-Phe-Gly) is located in a deep cleft between the two lobes; while the activation loop (in which the Thr^{778} residue is labelled) is situated near the entrance to the active site of the kinase. The V5 domain assumes an extended loop structure and transverses the surface of the two lobes. As shown in Fig. 3.9*A*, *B*, the extreme C-terminus of the V5 domain containing the hydrophobic phosphorylation motif makes a turn at the apex of the kinase core and embeds in the conserved hydrophobic patch that is located at the back of the kinase core, as in the case of other AGC kinases [85, 338].

To define the possible roles of the V5 domain of PRK1 in maintaining the active conformation(s), I focused on the carboxyl terminal residues 911-946. This stretch of sequence shows low sequence homology among the V5 domains of various AGC kinases and assumes variable conformations in different protein kinases. In the absence of a crystal structure, I used molecular dynamics (MD) to refine my homology to provide an estimate of its stability. The root mean square deviation (RMSD) from the initial protein model was analysed, focusing on intramolecular distance between residues Gln^{690} in the β4-sheet in N-lobe and Phe^{936} within the hydrophobic motif in the V5 domain. The δ-carboxyl group of Gln^{690} was found to form a hydrogen bond with the carbonyl group of Phe^{936} in my analysis. The intramolecular distance between Gln^{690} and Phe^{936} is shown in Fig. 3.10*A*. In a stable protein, the intramolecular distances fluctuate around an equilibrium, but in a non-equilibrated conformation there will be a continuous drift in the intramolecular distances as the MD protocol samples the conformational space for its global minima. The RMSD of the intramolecular distance between Gln^{690} and Phe^{936} was found to

drift towards a stable value of about 8.5 Å after 500 ps, suggesting that the atomic model of rPRK1 built using homology modeling (Fig. 3.9 *B-D*) is energetically stable. Most of the protein motions were found to be confined to the carboxyl terminus and the unstructured loops (Fig. 3.10*B*). The conformation of the protein model remained relatively stable after the initial 500 ps and the coordinates of $rPRK1_{612-946}$ at the end of the 5 ns simulation were used as a basis for predicting the potential interactions between residues in the hydrophobic motif in the V5 domain and those in the N-lobe (Fig. 3.9*D*). The model from my simulation shows that Asp^{940} potentially forms salt bridges with Lys^{668}, whereas the δ-carboxyl group of Gln^{690} and the ε-amino group of Lys^{668} independently formed a hydrogen bond with the carbonyl group of Phe^{936}. Phe^{939}, Phe^{936}, Phe^{689}, and Val^{695} potentially form a hydrophobic cluster (encircled with *light blue dotted line* in Fig. 2.9*D*) that would help to stabilize the N-lobe. Val^{942}, Phe^{941} and Met^{665} may form hydrophobic contacts (encircled with *pink dotted line* in Fig. 3.9*D*).

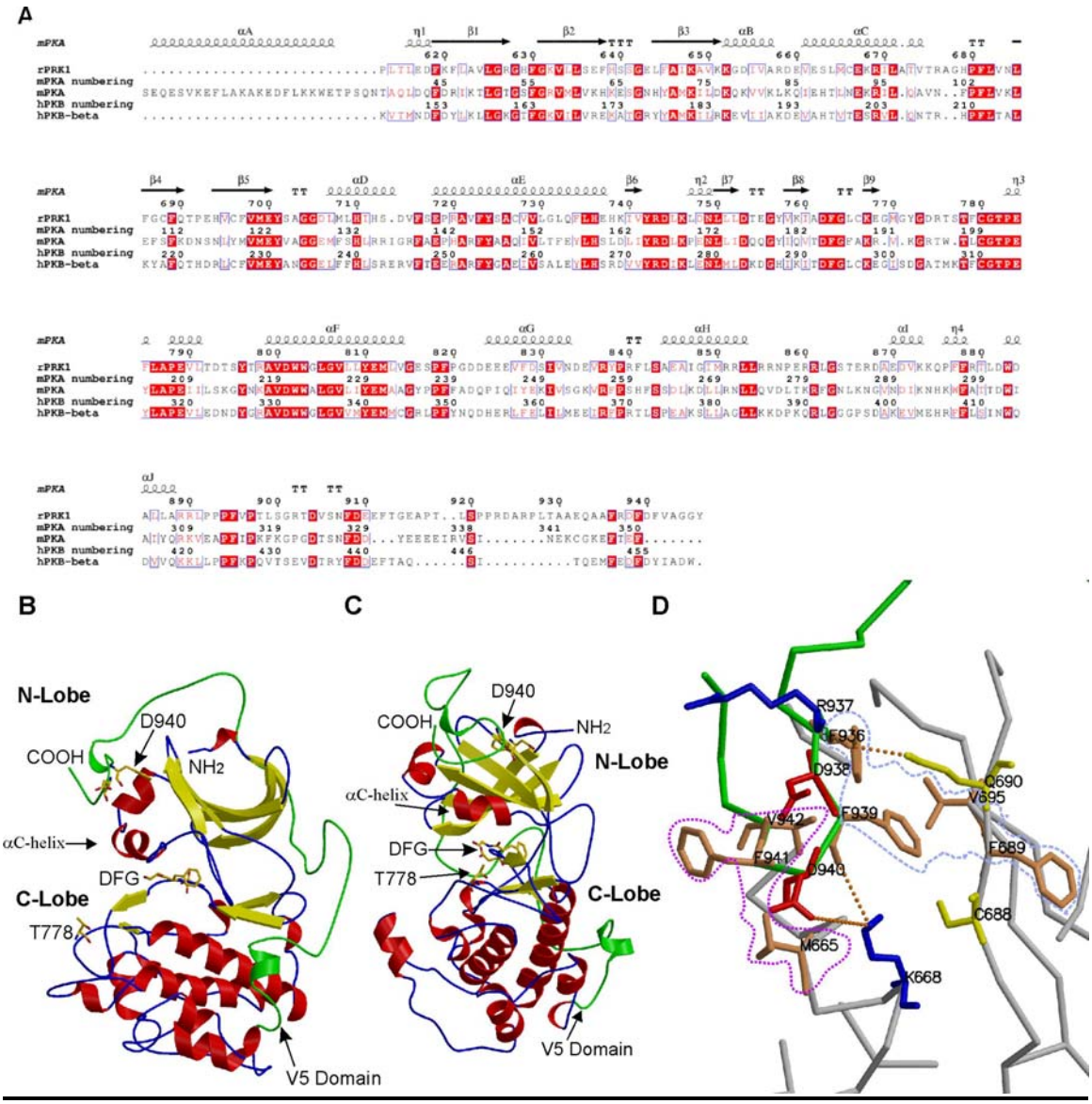


Fig. 3.9. Homology model of PRK1 catalytic domain. *A*, alignment of the catalytic domains of rat PRK1 (rPRK1) with mouse PKA (mPKA, Protein Data Bank code 1APM) and human PKBβ (h PKBβ, Protein Data Bank code 1O6L). The number on top of the alignment refers to sequence of rat PRK1 with the numbering of mouse PKA and human PKBβ labelled above the respective sequences. Identical residues are shown by white letters on a red background, while similar residues are denoted by red letters in blue boxes. The secondary structure is shown as black arrows for β strands and as loops for α helices. Note, in the engineered human PKBβ (1O6L), the last 15 amino acid residues were replaced by a PIFtide from PRK2 (21). *B*, a ribbon model of residues 612-946 of rPRK1. α-helices are shown in *red*, extended β strands are colored *yellow* and unstructured loops are shown in *blue*. The V5 domain is denoted in *green*. Asp-940 in the conserved hydrophobic motif, the DFG ATP-binding motif and the conserved PDK-1 phosphorylation residue Thr-778 of the activation segment are shown as *sticks*. Figures were drawn using POVScript+ (http://www.stanford.edu/~fenn/povscript/). *C*, a 90° rotation of *B*. *D*, a close up

view of the hydrophobic motif and the residues interacting with the N-lobe. The V5 domain is shown in *green*. Backbones are shown in *grey*. Residues shown as sticks are colored as follows: hydrophobic residues in *brown*; acidic residues in *red*; basic residues in *blue* and polar residues in *yellow*. Asp-940 potentially forms salt bridges with Lys-668 and Phe-936 potentially forms a salt bridge with Gln-690 (indicated as *brown dotted lines*). The δ-carboxyl group of Gln-690 and the ε-amino group of Lys-668 independently formed a hydrogen bond with the carbonyl group of Phe-936 (*brown dotted lines*). Phe-939, Phe-936, Phe-689, and Val-695 form a hydrophobic cluster (encircled with *light blue dotted line*) that helps to stabilize the N-lobe. Val-942, Phe-941 and Met-665 represent hydrophobic contacts (encircled with *pink dotted line*).

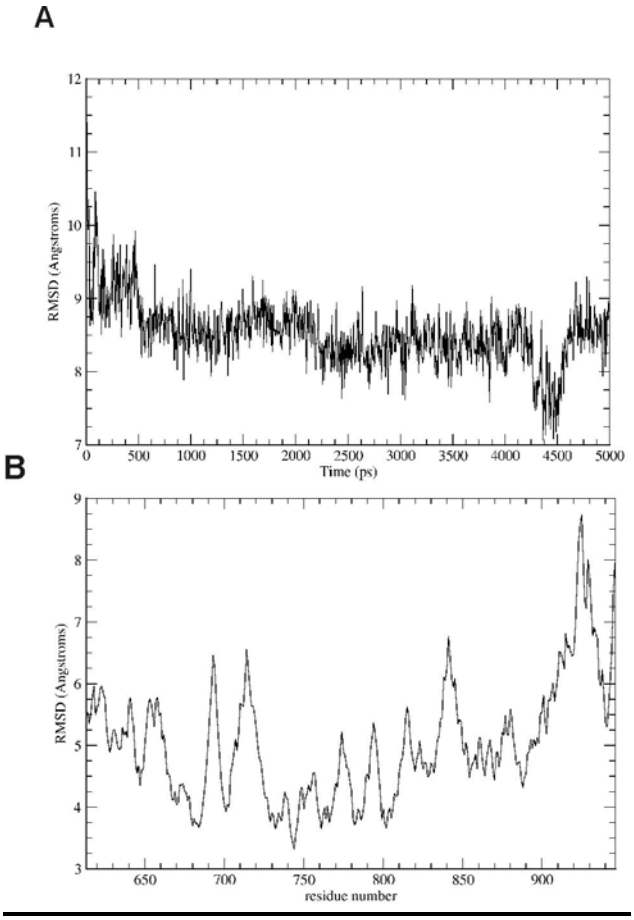


Fig. 3.10. **Molecular dynamics simulation of catalytic domain of PRK1**. *A*, C_α RMSD of the intramolecular distance between Q690 and F936 was analyzed as a function of time over the entire trajectory of a 5 ns molecular dynanmics simulation for $rPRK1_{612\text{-}946}$. *B*, the main chain (N, C_α, C and O) RMSD for each residue as a function of time during the same molecular dynanmics. α-helical sections of the structure are highlighted in *red* whereas extended strands are highlighted in *yellow*.

3.2 DISCUSSION

One of the key questions in PKC research is how the exquisite PKC isozyme specificity is achieved *in vivo*, given that members of the PKC superfamily are seemingly catalytically promiscuous when studied *in vitro*. Structurally, all known members of the PKC superfamily, except for the recently reported PKCζII [339], are composed of a highly conserved catalytic core (C3-V4-C4 domains) flanked by an less conserved NH_2-terminal regulatory segment (V1-C1-V2-C2-V3 domains) and a C-terminal V5 tail. The catalytic core of PKC seems less likely to contain determinants for *in vivo* isozyme specificity. Although the NH_2-terminal regulatory domain of the PKC superfamily kinases certainly plays major roles in allosteric regulation, how this domain contributes to the known isozyme-specific functions within subgroups of PKC superfamily remains to be explored. The V5 domain is a segment of approximately 50-70 amino acid residues located C-terminal to the catalytic core of PKC. Despite the fact that it contains several highly conserved residues, it apparently has the lowest overall amino acid sequence identity in PKC [340]. Such low conservation of sequence homology led some investigators to propose that the C-terminal portion of PKC has little importance in PKC function [341]. However, more recent studies show that the V5 domain is not only important in the maturation of PKC [45] but also governs some of the PKC's isozyme-specific functions [120, 342].

Based on the results of expression of C-terminal deletion mutants of bovine PKCα in the yeast [341], one might predict that the removal of the V5 domain of PKC could generate a mutant kinase that is insoluble [45]. However, at least in the case of PRK1, removal of up to 94% of the V5 domain (PRK1-Δ882) still resulted in a

mutant kinase with substantial solubility (~40% of that of the wild-type enzyme). Further deletion of the V5 domain of PRK1 (PRK1-Δ881) resulted in diminished solubility of the mutant kinase. Intriguingly, additional deletion into the catalytic core of PRK1 (PRK1-Δ860) generated a mutant kinase with enhanced solubility (Fig. 3.2). My data indicate that both the NH_2-terminal portion of the V5 domain and the C-terminal portion of the catalytic core are important for the proper folding of PRK1.

Although the C-terminal extension segment in the V5 domain of PRK1 (Asp^{940}-Tyr^{946}) is dispensable for the solubility of the kinase, this segment is important for the stability of PRK1. Removal of this carboxyl tail in the V5 domain resulted in a ~ninefold decrease of the half-lives of the PRK1-Δ941/940 mutants when introduced into COS-1 cells (Fig. 3.5 and Table 3), suggesting that this segment may guard PRK1 against premature degradation by cellular proteolysis machineries such as the proteasome system [343]. In terms of the contribution to the catalytic competence of the kinase, my results show that the removal of this segment has little, if any, appreciable effect on the catalytic activity of the mutant PRK1-Δ940 under my experimental conditions (Fig. 3.3*A*). However, once a stop codon was placed at the position of Phe^{939}, which corresponds to the C-terminus of PKA, the catalytic activity of the resultant PRK1-Δ939 was completely lost. The sharp demarcation of the catalytic activity between PRK1-Δ940 and PRK1-Δ939 mutants is surprising: instead of a quantitative change in the catalytic activity, an all-or-none effect was observed with deletion of just one additional amino acid residue.

One of the key recent advances in the study of the regulation of the PKC superfamily of serine/threonine kinases is the elucidation of the importance of turn motif and

hydrophobic motif in the maturation, signaling and down-regulation of PKCs [45, 112, 198]. Although the role of phosphorylation at these two motifs in the regulation of PKC has been well defined with selected PKC isozymes, the contribution by the phosphorylation at these two motifs to PKC isozyme specificity is less well understood. An emerging theme regarding the presence of these two motifs in all PKC isozymes is their unique contribution to the biochemical properties of each isozyme. For turn motif, the mutation of the phosphor-acceptor residue to alanine in closely related classic PKC (cPKC) isozymes reveals strikingly different contribution of the phosphothreonine to the catalytic activity of individual cPKC: the PKCβ1-T642A mutant has completely lost its catalytic activity [344], while PKCα-T638A mutant has retained ~80% of the catalytic activity of the wild-type counterpart [103]. As for novel PKC (nPKC), the serine-to-alanine mutant in turn motif of PKCδ (PKCδ-S643A) retains ~53% of the specific activity of the wild-type enzyme [104]. In contrast, it is demonstrated in this study that the PRK1-S920A mutant had low catalytic activity (Fig. 3.4). It remains to be established whether the observed different effects of turn motif threonine/serine-to-alanine mutation on the specific activity of PKC isozymes are due to different degrees of compensating phosphorylation of surrounding serine/threonine residues [130], selective interaction with other cellular proteins such as heat shock proteins [212] or different types of host cell lines used for the production of PKC mutants. In terms of the hydrophobic motif at the V5 domain, the effect of serine-to-alanine mutant on PKC's specific activity is drastically different for closely related cPKC isozymes: PKCβ2-S660A has greatly reduced catalytic activity [130] but PKCα-S657A mutant has very similar catalytic activity to the wild-type protein [129]. In atypical PKC (aPKC) and PRK subgroups, the phosphor-acceptor Ser/Thr residue in hydrophobic motif is replaced by an acidic

residue, aspartic acid or glutamic acid. Nonetheless, isozyme-specific effects of Glu/Asp-to-alanine mutation persist. A PKCζ-E579A mutant retains 55% specific activity compared with the wild-type enzyme, PRK2-D978A-ΔNT (generated on a version of PRK2 that lacks the auto-inhibitory N-terminal domain) is only 20% active compared with the wild-type enzyme [345]. In contrast, I show here that the PRK1-D940A mutant is completely inactive under my assay conditions (Fig. 3.4). The data presented here reinforce the notion that each member of the PKC superfamily must be studied individually to reveal isozyme-specific variation of the common theme of regulation and to understand the mechanistic basis for isozyme-specific function of PKC *in vivo* [112, 346].

Using a strategy combining serial deletion and site-directed point mutation, I made several intriguing observations on the role of V5 domain in maintaining a catalytic competent conformation of PRK1. First, deleting the C-terminal extension (940-946) of the V5 domain in PRK1 had no apparent negative effect on the *in vitro* catalytic competence of the PRK1-Δ940 and PRK1-Δ941 mutants. In contrast, replacing the Phe^{941} with alanine in the full-length PRK1 resulted in ~80% reduction of the catalytic activity of the PRK1-F941A mutant (Fig. 3.3*A*). Second, mutation of the last amino acid residue (Phe^{939}) in PRK1-Δ940 (including PRK1-Δ940-F939W) resulted in an inactive kinase (Fig. 3.3*C*); while the same Phe^{939}-to-Trp mutation introduced into the full-length protein had only a moderate effect on the catalytic activity of PRK1-F939W mutant (~55% of the wild-type protein) (Fig. 3.4*A*). Third, the PRK1-D940A mutant was catalytically inactive (Fig. 2.4*A*); while deleting the Asp^{940} and the rest of the C-terminal residues had little effect on the catalytic activity of the resultant PRK1-Δ940 mutant (Fig. 3.3*A*). Similar intriguing observations have been

made for PKA: while a PKA mutant with a Phe^{350}-to-Ala mutation is devoid of measurable enzymatic activity (51), the PKA deletion mutant lacking C-terminal 16 amino acid residues retains 40% activity [347]. It is possible that the C-terminal extension of the V5 domain in PRK1 (residues 940-946) interacts with other elements in PRK1. Such interactions may be important for regulatory functions but might not be required for the catalytic competence of the kinase *per se*. In fact, the net outcome of such interactions might put unfavourable structural constrains on active conformations of the kinase and therefore necessitate the presence of a negatively charged residue in position 940 (Asp^{940}) to override the unfavourable constrains in the wild type enzyme. It is the collective intramolecular interactions between residues in the C-terminus and those in the rest of the protein that define an active conformation of PRK1 and ensure isozyme-specific regulatory flexibility. This hypothesis is supported by my model of the three-dimensional structure of the catalytic domain of PRK1 based on homology modelling and molecular dynamics simulation. In my homology model of PRK1 (Fig. 3.9), Phe^{941} and Val^{942} potentially form hydrophobic interaction with Met^{665} located at αC helix; while Asp^{940} potentially forms a salt bridge with Lys^{668} (corresponding to Lys^{92} in PKA) at αC helix. The αC helix in PKA and PKB has been shown to be instrumental in organizing an active kinase structure by maintaining the nucleotide-binding site and activation segment in a catalytically competent state and integrates inputs from other segments in the catalytic core [85, 107, 111]. Importantly, the extent of the twist of αC helix has been shown to determine the functional mode of protein kinases [338, 348] and the precise position of αC helix can be determined by the phosphorylation state of the protein or the binding of nucleotide [85, 111]. The result from the homology modeling suggests an explanation on the paradoxical result where D940A mutant was catalytically

inactive but PRK1-Δ940 has high activity. Replacing aspartate with a non-polar residue of Ala may play an inhibitory role, such that alanine at position 941 interferes with the hydrophobic interaction between the aromatic residues of Phe^{939} and Met^{665}. At the same time, the findings that mutation of PRK1-F949Y has less effect than deleting the residue together with the rest of the C-terminal residues suggest that tryptophan in place of phenylalanine, together with phenylalanine at 941 largely maintains the configuration required for kinase activity. Thus, it is possible that in the full-length PRK1, the side chains of the last 6 amino acid residues at the C-terminus interact with the αC helix in such a way that elements in the catalytic core are not optimally aligned. The additional contribution from the side chain of Asp^{940} might help to orient αC helix properly to allow the final configuration of an active conformation of PRK1 through its interaction with Lys^{668} and other residues in the αC helix. Such a critical coordinating function of Asp^{940} would become redundant for the catalytic competence in the absence of the last 6 amino acid resides as in the case of the PRK1-Δ940 mutant.

Recently, a hydrophobic pocket located at the back of the N-lobe of AGC kinases has been identified [85, 270, 349]. This pocket is formed by the hydrophobic residues from αB and αC helices with the β4 and β5 strands of the central β-sheet. The interaction of the hydrophobic residues at the C-terminus of AGC kinases with this pocket stabilizes the ordered αB and αC helices and thus is essential for catalysis. In my model of PRK1, the Phe^{936} and Phe^{939}, (corresponding to Phe^{347}/Phe^{350} in PKA or Phe^{470}/Phe^{473} in PKB) potentially forms hydrophobic contact with Phe^{689} (in β4 strand) and Val^{695} (in β5 strand). Tempering Phe^{939} with an alanine substitution or

deletion will therefore inevitably compromise the conformation required for a catalytically active kinase.

A major leap in the understanding of how PKC is regulated *in vivo* was the discovery of PDK-1 as the upstream kinase that phosphorylates the activation loop of PKC in 1998 [97, 98, 100]. Most members in the PKC superfamily of serine/threonine kinases, except for PKCδ, are synthesized as an inactive precursor in cells [45]. The phosphorylation of a threonine residue within the context of a conserved Thr-Phe-Cys-Gly-Thr motif in the activation loop located in the kinase subdomain VIII of PKCs constitutes the initial priming step that triggers a maturation process that is mandatory for most of the PKCs to become a functional kinase [45, 198, 350]. Studies of the role of hydrophobic motifs in the regulation of PKCζ and PRK2 by PDK-1 via mutagenesis analysis show that mutation of glutamic acid or aspartic acid or any of the conserved hydrophobic residues in the hydrophobic motif (Phe-Xaa-Xaa-Phe-Ser/Thr/Asp/Glu-Phe/Tyr) either abolished or greatly reduced the ability of PDK-1 to interact with PKCζ and PRK2 and to phosphorylate the activation loop [345]. Such observations led to a generalized proposal advocating that the hydrophobic motif of AGC kinases functions as a substrate-docking site to enable PDK-1 to bind and then phosphorylate its substrates [270]. However, findings from this study suggest that careful dissection of the contributions from individual residues in the hydrophobic motif and those from other domains of the kinase in question is necessary for a complete and comprehensive picture of how PDK-1 interacts with individual isozymes in the PKC superfamily. First, my study has shown that a PRK1-Δ940 deletion mutant lacking the last two residues in the hydrophobic motif is catalytically active and with the activation loop well phosphorylated (Fig. 2.5, Fig.

2.6). Hence, at least in the cases of PRK1, the last two residues in the hydrophobic motif are dispensable for interaction with PDK-1. In fact, hydrophobic motif originated as a hydrophobic phosphorylation motif (FSF/Y) where serine is flanked by two hydrophobic residues. In the case of aPKC and PRK, the phosphor-acceptor is replaced by an negatively charged Glu/Asp residue) [45]. Therefore, if one deletes the phosphor-acceptor residue and its C-terminal hydrophobic residue, the hydrophobic phosphorylation motif would no longer exist. In this sense, the hydrophobic motif in both PRK1 is not required for the docking and phosphorylation of the activation loop by PDK-1. Second, my serial deletion experiments showed that PDK-1 could still physically interact with PRK1-Δ770 even after the removal of a segment encompassing the activation loop and the C-terminus of PRK1 (Fig. 3.7). Third, domains in PKC other than the catalytic core can influence the interaction of PDK-1 with PKC. For example, when expressed in HEK293 cells, the catalytic domain-only construct of PKCζ is not phosphorylated at Thr^{410} in the activation looop. However, the full-length PKCζ-F578A mutant is partially phosphorylated at Thr^{410} and has reduced but significant catalytic activity [351]. Furthermore, when introduced into tsA201 cells, the glutathione S-transferase (GST)-fusion full-length PKCβII associates well with the co-expressed PDK-1. However, the GST-fusion construct of the catalytic domain of PKCβII interacts with PDK-1 poorly in the same cells [110]. Thus, at least in the case of PKCβII and PKCζ, the regulatory domain of PKC may play a role in PDK-1 interaction. Fourth, in embryonic stem cells lacking PDK-1 (the PDK-1 gene knockout cells), there is a remarkable isozyme-specific difference in the detectable protein levels of members of the PKC superfamily: the expression of PKCζ and PRK2 is similar to that in control cells; while the expressions of many other PKC isozymes are reduced with virtually undetectable protein levels

for PKCε and PRK1 [352]. The amount of mRNA for PRK1 in PDK-1 null cells is the same as that in the control cells. The absence of PRK1 protein in PDK-1-null cells is unlikely to be due to the lack of phosphorylation of the activation loop of PRK1 by PDK-1, as in my hands the protein for a PRK1 mutant in which all the four possible phosphor-acceptor serine/threonine residues in the activation loop are replaced by alanine (PRK1-4A) is readily detectable when transfected into different types of mammalian cells (Fig.3.6 and data not shown). Rather, this indicates that PDK-1 may directly or indirectly regulate the biosynthesis and/or *in vivo* stability of some of its PKC substrates at a site other than the activation loop.

PKCs are lipid activated kinases [8]. With the exception of diacylglycerol and phorbol ester, there is limited knowledge about how other lipids, such as fatty acids, ceramide, phosphatidic acid and lysophospholipids, activate PKCs. For PRK1, the structural and biochemical basis for its activation by Rho proteins has been extensively characterized [56, 266, 353]. However, the mechanisms of PRK1 activation by other fatty acid and phospholipids [44, 53, 54] remain elusive. Ono and colleagues identified an essential autoinhibitory domain (residues 455-511) near the N-terminal of the V3 (hinge) domain of PRK1 [267]. It is proposed that arachidonic acid directly interferes with the interaction between this autoinhibitory domain and the catalytic domain, thus relieving the autoinhibition on PRK1 [267]. My data indicate that the last 5 amino acid residues at the C-terminus of PRK1 are essential for full lipid responsiveness. The activation of the PRK1-Δ942 mutant in response to the stimulation of 40 μM arachidonic acid was almost abolished (Fig. 3.8). To my knowledge, this is the first report on the identification of a modulating element of lipid responsiveness at the C-terminus of a member of the PKC superfamily of

serine/threonine kinases. The C-terminus of the V5 domain of PKC is a segment that displays great variability (in terms of amino acid sequence and length) among isozymes in the family. This small segment is ideally poised for conferring isozyme-specific regulation of each member in the PKC superfamily. For example, it has been shown that the last four amino acid residues at the C-terminus of PKCα are essential for the binding of PICK1 and consequently the mitochondrial targeting of PKCα in NIH3T3 cells upon serum stimulation [125, 354]. In *Drosophila* eye PKC, the last three amino acid residues at C-terminus have been shown to be critical in the binding of INAD and thus proper visual transduction in the fly [355, 356]. It has been well established that in mammalian cells, most arachidonic acid is esterified as a fatty acyl chain of glycerophospholipids in cell membranes, in particular the plasma membrane [357]. Free arachidonic acid is released in cells in response to a variety of cellular stimulations that result in the activation of phospholipases (phospholipase A_2, diacylglycerol and monoacylglycerol lipases) [358]. Our laboratory has recently identified an integral plasma membrane pool of PRK1 that transduces signals from activated RhoA [359]. It is therefore tempting to speculate that PRK1 could be activated by cellular stimuli that result in the release of free arachidonic acid from the plasma membrane to mediate physiological and pathological processes in which arachidonic acid has been shown to be involved, such as insulin secretion [360], neurotransmitter release [361], mood disorders/schizophrenia [362, 363], colorectal carcinogenesis [364] and cell death [365].

In conclusion, this study has shown that in PRK1, the seven amino acid residues at the C-terminal segment (which is absent in PKA) are an important element in conferring *in vivo* stability in PRK1. The full-length hydrophobic motif in PRK1 is dispensable

for catalytic activity and the interaction with PDK-1. PDK-1 can physically interact with PRK1 at the catalytic core in the absence of the V5 domain that harbours the hydrophobic motif. The last five amino acid residues at the C-terminus of PRK1 control the full lipid response of the kinase to arachidonic acid. Elucidation of the function of the C-terminal segment in members of the PKC superfamily of serine/threonine kinases will not only advance understanding of the biochemical mechanisms underlying the known *in vivo* isozyme specificity but also might pave the way for the development of highly selective pharmacological agents that are able to modulate the activity of each isozyme of PKC in human diseases.

Appendix D:

Sequencing Results of PRK1 mutants

>Rat PRK Δ946_sequencing primer DWS 68

TTNANTTTNAACNGACCNCCGTTGATCCNNANNCCNNCTNGNTAANTCGAGNTNGGGGCNTGAANGNTN
TTTATTCCCCNGGGNTTTNCCTAANCTNAAAAGGNNTTACCCGGAGNGTGNACCTATTNGAAAGGATTT
GNACCNNNCAGGCAGAATCCTNCTNNGAACNTTTGGGNNTNCANNCCCAAGCATTTTTTTGGATGATAT
TAGCCGNGGGACTGATTTGCANTNCACAGGACGTTTTTCAGANCTCGGNTNTTTCTATTCGCCTGTGTG
TGCTGGGACTGCAGTNCTCCATGAACACNAGATGTNTACAGGGACCTGAAGTTGGACAATTTGNTCCTN
GATACTGAGGGCTACGTCAAGATCGCAGACTTTNGCCTCTGCAAGGAGGGGATGGGCTATGGGGACCGG
ACCAGCACATTCTGCGGAACTCCGGAGTTCCTGGCGCCGGAAGTGCTCACAGACACATCCTACACTCGA
GCCGTGGACTGGTGGGGACTGGGTGTATTGCTCTATGAGATGCTGGTTGGAGAGTCTCCGTTCCCTGGG
GACGACGAGGAGGAAGTATTTGACAGCATCGTCAATGATGAGGTTCGTTATCCCCGCTTCCTGTCTGCG
GAGGCCATCGGCATCATGAGAAGGCTACTGCGGAGGAACCCAGAGCGGAGGTTGGGATCCACTGAGCGT
GATGCAGAAGATGTGAAAAAACAGCCTTTCTTCAGGACTCTGGACTGGGATGCCCTGCTGGCCCGTCGC
CTGCCTCCACCCTTCGTGCCTACACTTTCGGGGCGCACAGACGTCAGCAACTTCGATGAGGAGTTCACT
GGGGAGGCCCCCACACTGAGCCCTCCCCGGGATGCACGGCCCCTGACAGCTGCGGAGCAGGCGGCCTTC
CGGGATTTCGACTTTGTGGCAGGAGGCTAATAGCCCTAAGCCCCTGCCTTGCCCAAGAGTTCTTGGTTT
TTAAAAAAGCCTTTGGGAATTCCTGCAGCCCGGGGGATCCACTAGTCTAGAGGGCCCTATTCAANAGTG
TCACCTAAATGCTACATGCTCGCTAAGANAGCCTTCGACTNTGCCTTCTANNTGCCA

>Rat PRK Δ944_sequencing primer DWS 68

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CCCGGGGGGACNTGATGCTGCATTTCCCAAGNGACGGTTTTCANAGCCTCGGGCTNTTTTTTTTTNGCC
TGTGNGGTGCTGGGACTGCAGTTCTTCCATGAAACACAAGATTGTTTACAGGGACCTGAAGTTGGACAN
TTTGNTCCTGGATACTGAGGGCTACGTCAAGATCGCAGACTTTGNCTNTGCAAGGAGGGGATGGGCTAT
GGGACCGGACCAGCACATTTTGCGGAACTCCGGAGTTCCTGGCGCCGGAAGTGCTCACAGACACATCCT
ACACTCGAGCCGTGGACTGGTGGGGACTGGGTGTATTGCTCTATGAGATGCTGGTTGGAGAGTCTCCGT
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CTTGGTTTTTAAAAAAGCCTTTGGGAATTCCTGCAGCCCGGGGGATCCACTAGTCTAGAGGGCCCTATT
CTATAGTGTCACCTAAATGCTAGAGCTCGCTGATACAGCCTNCGACTNTGCCTTNTAGTNCCA

>Rat PRK Δ942_sequencing primer DWS 68

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GACTTTGGCCTNTGCAAGGAGGGGATGGGCTATGGGGACCGGACCAGCACATTCTGCGGAANTCCGGAG
TTCCTGGCGCCGGAAGTGCTCACAGACACATCCTACACTCGAGCCGTGGACTGGTGGGGACTGGGTGTA
TTGCTCTATGAGATGCTGGTTGGAGAGTCTCCGTTCCCTGGGGACGACGAGGAGGAAGTATTTGACAGC
ATCGTCAATGATGAGGTTCGTTATCCCCGCTTCCTGTCTGCGGAGGCCATCGGCATCATGAGAAGGCTA
CTGCGGAGGAACCCAGAGCGGAGGTTGGGATCCACTGAGCGTGATGCAGAAGATGTGAAAAAACAGCCT
TTCTTCAGGACTCTGGACTGGGATGCCCTGCTGGCCCGTCGCCTGCCTCCACCCTTCGTGCCTACACTT
TCGGGGCGCACAGACGTCAGCAACTTCGATGAGGAGTTCACTGGGGAGGCCCCCACACTGAGCCCTCCC
CGGGATGCACGGCCCCTGACAGCTGCGGAGCAGGCGGCCTTCCGGGATTTCGACTTTTAGGCAGGAGGC
TATTAGCCCTAAGCCCCTGCCTTGCCCAAGAGTTCTTGGTTTTTAAAAAAGCCTTTGGGAATTCCTGCA
GCCCGGGGGATCCACTAGTCTAGAGGGCCCTATTCTATAGTGTCACCTAAATGCTAGAGCTCGCTGATT
CAGCCTTCGACTGTGCCTTCTANTGCCAC

>Rat PRK Δ941_sequencing primer DWS 68

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>Rat PRK Δ940_sequencing primer DWS 68

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>Rat PRK Δ938_sequencing primer DWS 68

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>Rat PRK Δ940 F939L_sequencing primer DWS 68

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Chapter 4:

C-terminus of PRK1 is essential for RhoA activation

This work has been published in:

Lim, W.G., et al., *The very C-terminus of PRK1/PKN is essential for its activation by RhoA and downstream signaling.* Cell Signal, 2006. **18**(9): p. 1473-81.

4.1 RESULTS

4.1.1 Generation and Characterization of Deletion Mutants of PRK1

Previously, a novel element within the extreme C-terminus that controlled the lipid responsiveness of PRK1 to arachidonic acid was identified [366]. To determine if the element within the V5 is specifically responsive to only arachidonic acid, PRK1 C-terminal deletion mutants were studied for the response to another PRK1 activator: RhoA. A full-length rat PRK1 cDNA was cloned into the mammalian expression vector pcDNA3. As the study involved serial deletion of C-terminal residues of PRK1, the DNA sequence coding for Myc-tag (EQKLISEEDL) was incorporated at the N-terminal before the second amino acid residue of PRK1 to facilitate the identification and purification of the recombinant PRK1.

Previous work has revealed that GST fusion HR1a or HR1b can bind RhoA but GST-HR1c does not interact with RhoA [55, 56]. It has also been shown that HR1abc binds RhoA more tightly than HR1a or HR1b alone [56, 353]. The focus of those studies was on the interaction of PRK1 and RhoA. Therefore, the authors removed the kinase domain and used only the N-terminal regulatory domain in their studies [55, 353]. In contrast, the present study aims to determine the contribution of the C-terminus of PRK1 to the response of PRK1 to RhoA stimulation. Serial deletion mutants were generated by introducing an in-frame stop codon at designated positions at the C-terminus of wild-type N-terminally Myc tagged rat PRK1 (Fig. 4.1A). For example, as illustrated in Fig. 4.1*B*, the PRK1-Δ941 mutant carried a stop codon at residue 941 with the last expressed residue being residue 940.

The HR1 region in the regulatory domain has been proposed to be the sole determinant for the interaction between PRK1 and RhoA, thus a negative control which does not respond to RhoA stimulation was generated by deleting the HR1 domain of PRK1. This was accomplished by deleting residues 30 to 280 from an N-terminally Myc-tagged rat PRK1 (Fig. 4.1*C*). Breifely, HR domain deletion mutants were generated with a pair of non-complimentary phosphorylated primrs that flank upstream of residue 30 and downstream of residue 280. The PCR product was ligated to repair the nick before transforming into *E. coli* and subsequent steps are as listed in Materials and Methods. This HR1 deletion mutant was named PRK1-ΔHR1 (Fig. 4.1*C*). To provide evidence that PRK1 C-terminal deletion is RhoA specific, serial deletion mutants were generated by introducing an in-frame stop codon at designated positions at the C-terminus of the HR1 deletion mutant. As illustrated in Fig. 4.1*B*, for example, the ΔHR1-Δ941 mutant carried a stop codon at amino acid residue 941 with the C-terminus of the mutant being amino acid residue 940 on a background of HR1 deletion (Fig. 4.1C). Since I reported previously that the C-terminal deletion mutants (PRK1-Δ941 and PRK1-Δ943) have an apparent half-life of approximately 6 h compared to that of 64 h for the wild-type PRK1 [366], the half-life of the HR1 deletion mutants in cells was measured. As shown in Table 4.1, the deletion of the HR1 region, either alone or in combination with C-terminal deletion, led to a further decrease of the apparent half-life of the PRK1-ΔHR1 mutants, presumably due to the accelerated rate of turnover of the mutant PRK1 proteins. However, such decreased half-life did not translate into significantly altered steady-state protein levels of the PRK1 deletion or deletion mutants in COS cells (Fig. 4.2). This means the amount of intact proteins that can be immunoprecipitated from each of the different deletion

constructs would be similar, thus I proceeded with the analysis of the activation of PRK1 mutants by RhoA.

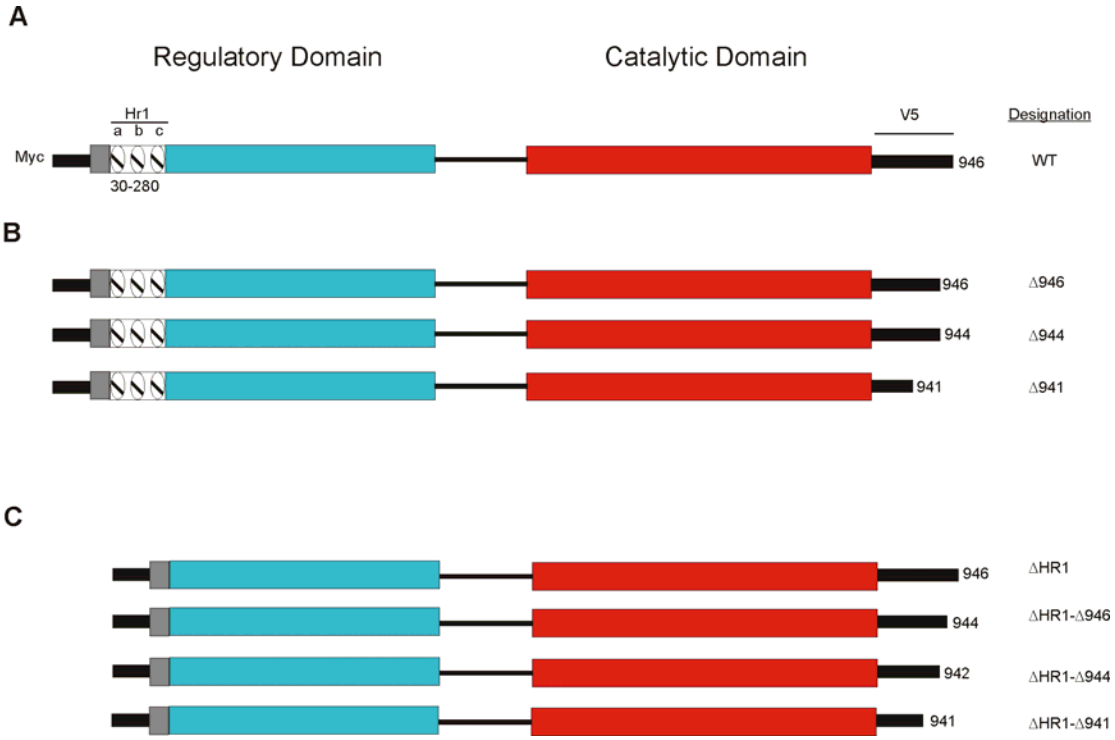


Fig. 4.1. Domain structure of PRK1 and PRK1 constructs used in this study. ***A***, Wild-type PRK1 (WT) is comprised of a regulatory domain (blue) and a catalytic domain (red) linked by a hinge region and was N-terminally tagged with a Myc epitope. The HR1abc region in the regulatory domain and the V5 domain is shown. The number at each C-terminus indicates the position of the stop codon, with the last expressed residue being the one just prior to the number. ***B***, The PRK1 C-terminal deletion mutants. *C*, The PRK1 N-terminal HR1abc and C-terminal deletion mutants.

TABLE 4.1

	WT-PRK1	ΔHR1	**Δ**HR1-Δ946	**Δ**HR1-Δ944
Half-life (h)	64 ± 5	2.1 ± 0.1	2.6 ± 0.15	2.25 ± 0.25

Apparent half-life of PRK1 and HR1-deletion mutants in COS cells

Values given as mean ± S.E. (n=4). COS-1 cells transfected with plasmids encoding wild-type (*WT*) or various deletion/deletion mutants of PRK1 were treated with 200 μg/ml cycloheximide and lysed hourly up to the 10 h time point. The amount of PRK1 in the total cell lysate at each time point was determined by Western blot analysis after normalizing the signals for β-actin. The apparent half-life was determined from plotting the amount of protein remaining against time on a semi-logarithmic graph as described under "Materials and Methods."

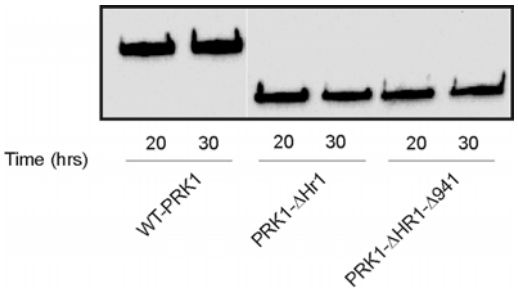


Fig. 4.2. Similar steady state of protein levels between wild type and deletion mutants. Myc-tagged wild type PRK1 and deletion mutants were transiently transfected into COS-1 cells. Twenty and thirty hours post transfection, cells were lysed and exogenous wild type or mutant PRK1 were immunoprecipitated with anti-Myc antibodies. The amount of protein immunoprecipitated was determined by Western blotting.

4.1.2 Effect of Deletion of HR1 on the Interaction between PRK1 and RhoA

Although previous studies have reached the conclusion that the HR1 region in PRK1 is the RhoA binding site, it is noteworthy that the studies were carried out using PRK1 constructs without the catalytic domain. In this study, the aim was to determine the contribution of the C-terminus to catalytic activity by RhoA activation in a more physiological setting, thus I choose to include both the regulatory and catalytic domains in my studies. The contribution of HR1 to the binding of a full length PRK1 to RhoA *in vitro* was first assessed. GST-fusion RhoA was produced using an *E. coli* expression system. The solubility and the amount of protein expressed were verified by Coomassie staining (Fig. 4.3A). It has been shown that PRK1 interacts with GTP-RhoA but not with GDP-RhoA in cells [57, 58], thus after affinity purification (Fig. 4.3B), the GST-RhoA was loaded with GTPγS, a GTP analogue that is not hydrolysable by GTPase to produce GTP-bound RhoA. Transfection of plasmids encoding the wild-type PRK1, the HR1 deleted PRK1, the C-terminal deletion or combined N- and C-terminual deletion mutants of PRK1 was carried out in COS-1 cells. The Myc epitope-tagged PRK1 was immunoprecipitated and stringently washed with 0.5M LiCl to remove non-specifically bound proteins. The purified Myc-tagged

PRK1 and GST-GTPγS-RhoA were incubated together for one hour to allow binding *in vitro*. Unbound RhoA was removed by three washes consisting of 1% NP-40 and 150 mM NaCl. The immunoprecipitated Myc-PRK1 was then subjected to SDS-PAGE and the interaction of GST-RhoA with Myc-PRK1 was determined by Western blot analysis to detect the co-immunoprecipitated GST-RhoA. The specificity of the binding assay was verified in the separate negative control experiments. First, Myc-PDK1 failed to co-precipitate GST-RhoA (Fig. 4.4A, last lane), demonstrating that GST-RhoA could not be co-precipitated by an over-expressed Myc-tagged protein in the experimental setting. Second, GST-Tau1 could not co-precipitate Myc-PRK1 or Myc-PRK1-ΔHR1 (Fig. 4.4C); demonstrating that a large amount of irrelevant GST-tagged protein was unable to co-precipitate Myc-PRK1.

As shown in Fig. 4.4*A* and 4.4*B*, the deletion of one to six residues from the C-terminus had only marginal adverse effects on the binding of PRK1 to RhoA. On the other hand, the deletion of the HR1 significantly reduced the amount of GST-RhoA co-immunoprecipitated with PRK1. Interestingly, the removal of the HR1 region from PRK1 did not completely abolish the interaction between PRK1 and GST-RhoA, as there was approximately 20% GST-RhoA co-immunoprecipitated with PRK1-ΔHR1 compared with that of the wild-type PRK1 (Fig. 4.4*B*). The deletion of the C-terminal residues from the PRK1-ΔHR1 mutant had no apparent additional effect on RhoA binding in the C-terminal deletion mutants of PRK1-ΔHR1 (Fig. 4.4*A* and *B*). Taken together, the results suggest that RhoA may interact with PRK1 directly at regions other than HR1.

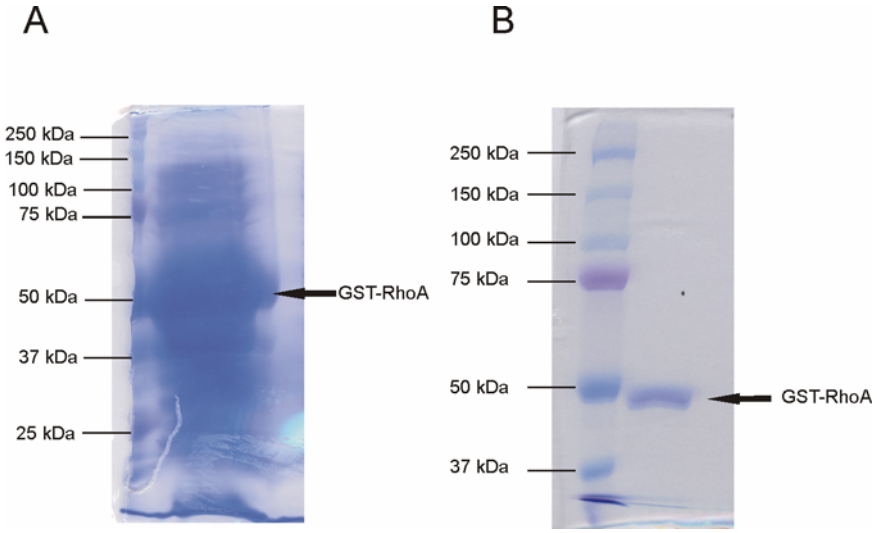


Fig. 4.3. Coomassie blue staining of bacterially expressed GST-RhoA. (A) Bacterial lysate before purification. **(B).** Affinity purified bacterial lysate showing single GST-RhoA band without other protein contamination.

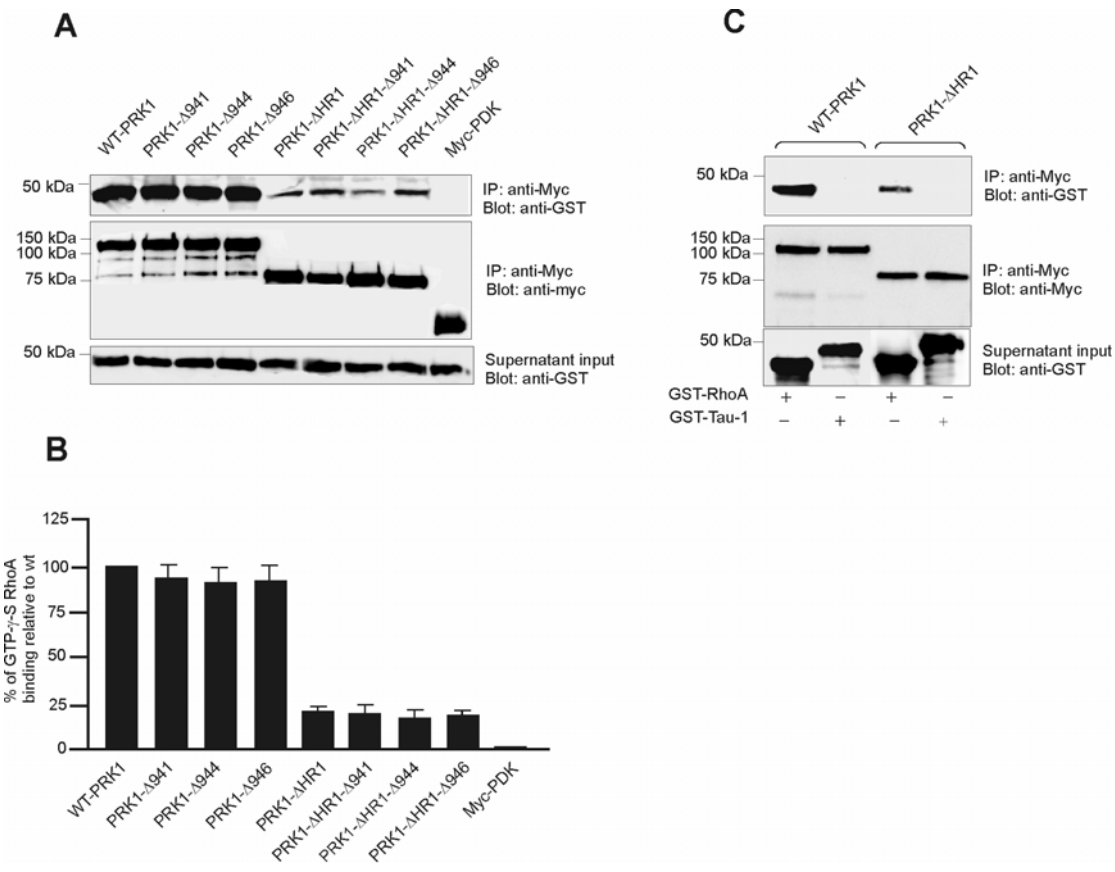


Fig. 4.4. ***In vitro*** **binding of GST-GTPγS-RhoA to PRK1 and its deletion-deletion mutants**. ***A***, PRK1 immunoprecipitation. Myc-tagged PRK1 constructs were expressed in COS-1 cells and immunoprecipitated followed by three stringent washings. Bacterially expressed GST-RhoA was loaded with GTPγS and then added to the immobilized PRK1. After incubation for 1 h, PRK1 was immunoprecipitated followed by two washings with 150 mM NaCl and 1% Triton X-100 as described under "Materials and Methods". The RhoA bound to immunoprecipitated PRK1 was

determined in Western blot analysis using an anti-GST antibody (*top panel*). The amounts of immunoprecipitated Myc-tagged PRK1 were determined using an anti-Myc antibody (*middle panel*). Equal amounts of input GST-GTPγS-RhoA protein were used (*bottom panel*) for PRK1 pull-down. ***B***, quantification of GST-GTPγS-RhoA bound to immunoprecipitated PRK1 *in vitro* as shown in *A*. Results shown are mean ± S.E. of at least five independent experiments and are represented as percentage of the GST-GTPγS-RhoA bound to the wild-type PRK1. ***C***, GST-Tau-1 was used to demonstrate that the interaction between GST-RhoA and PRK1 is specific in PRK1 pull-down. Bacterially expressed GST-Tau-1 or GST-GTPγS-RhoA was used for PRK1 immunoprecipitation. The GST-protein co-immunoprecipitated (*top panel*) and the immunoprecipitated PRK1 (*middle panel*) were determined in Western blot analysis using anti-GST and anti-Myc antibodies, respectively. Amounts of input GTP-fusion protein in each sample are shown in the *bottom panel*. Data shown are representative of at least five (Fig. 4.4*A*) or two (Fig. 4.4*C*) independent experiments.

4.1.3 Contribution of Regions other than HR1 to the Activation of PRK1 by RhoA *in vitro*

Having demonstrated that HR1 is not the sole region responsible for RhoA binding with PRK1 *in vitro*, a question arose as to whether this putative new Rho binding site is functional in activating PRK1. In other words, is HR1 the only element on PRK1 that is required for the activation of this kinase by RhoA? To assess the direct interaction and activation of PRK1-ΔHR1 by RhoA, *in vitro* kinase assay was performed to examine changes in the kinase activity of PRK1-ΔHR1 in the presence of RhoA. To this end, PRK1 deletion mutants were over-expressed in COS cells and immunoprecipitated. Immunoprecipitated PRK1 mutants were equally divided into two tubes to ensure similar amount of proteins were used. Subsequently, PRK1 deletion mutants were tested for their response to GTPγS-RhoA in an *in vitro* immune-complex kinase assay employing an S6 peptide as a substrate in the presence or absence of the activator GTPγS-RhoA. Ideally, the negative control for this RhoA activation experiment would be a PRK1 protein which does not interact with RhoA. However, as shown in Fig. 4.4A, HR1 deleted PRK1 is still capable of binding RhoA *in vitro*. Thus, a surrogate negative control of PRK1 with activation loop mutation

(4A) is used instead as this mutant is kinase inactive and would not respond to activation.

Consistent with previous reports on the activation of PRK1 by RhoA *in vitro* [57, 58], in the *in vitro* system of this study, the addition of GST-GTPγS-RhoA to the immuno-purified wild-type PRK1 resulted in an approximately 4-fold activation of PRK1 as determined by its capacity to phosphorylate an exogenous S6 peptide (Fig. 4.5*A* and *B*). Surprisingly, the removal of only one amino acid residue from the C-terminus of PRK1 resulted in an approximately 40% reduction of the activation (Fig. 4.5*A*). Further deletion of two to six residues from the C-terminus resulted in progressive reduction of the magnitude of RhoA activation in the PRK1 C-terminal deletion mutants, culminating at the PRK1-Δ941 mutant that was hardly activated by RhoA (Fig. 4.5*A*) despite the fact that it could physically interact with GST-RhoA (Fig. 4.4*A* and *B*). Even more surprising was the finding that the deletion of the HR1 region resulted in a PRK1-ΔHR1 mutant that could still be activated by GTP-RhoA for about 2-fold (Fig. 4.5*B*). The deletion of one or more C-terminal residues from the PRK1-ΔHR1 mutant completely abrogated the activation of the mutant PRK1 by RhoA (Fig. 4.5*B*). These findings indicate that the HR1 region is only partially responsible for the direct activation of PRK1 by RhoA *in vitro*. It is the very C-terminus of PRK1 that critically controls the direct activation of this kinase by RhoA, at least *in vitro*.

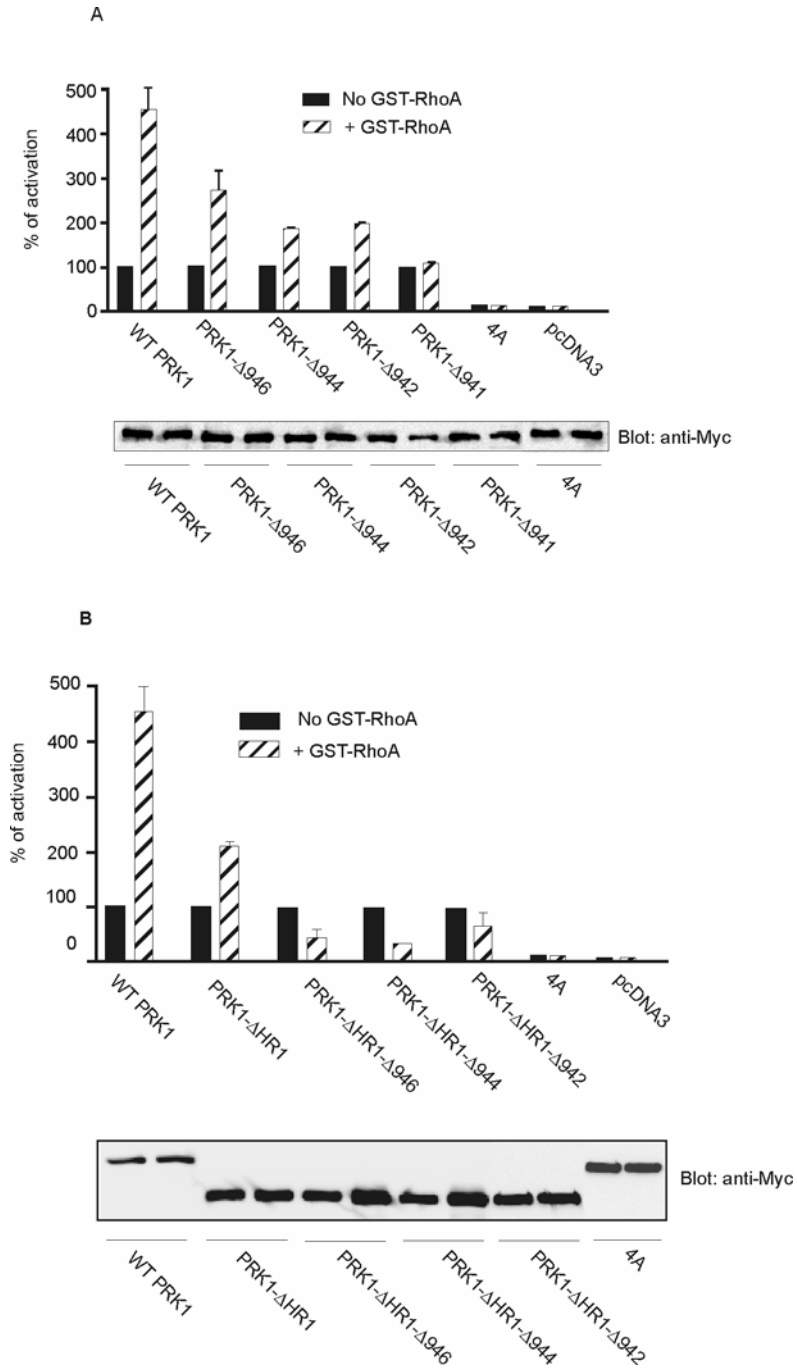


Fig. 4.5. *In vitro* activation of PRK1 by GST-GTPγS-RhoA. The catalytic activity of wild-type PRK1 and that of PRK1 C-terminal deletion mutants (*A*) or HR1-deletion and HR1-deletion plus C-terminal deletion mutants (*B*) was determined in the presence or absence of GST-GTPγS-RhoA. A S6 peptide was used as a substrate in the transphosphorylation assay as described under "Materials and Methods". The amount of PRK1 or PRK1 mutant protein captured on beads during kinase assay in each sample was determined in a Western blot analysis using an anti-Myc antibody to derive specific activity of PRK1. Data shown are mean ± S.E. of at least three to five independent experiments and are represented as percentage of the activity of the corresponding PRK1 or PRK1 mutant that was not treated with GST-GTPγS-RhoA. A representative Western blot used to derive the specific activity for each pair of samples is shown below each bar chart.

4.1.4 Critical Role of the C-terminus of PRK1 in its Activation by RhoA in Cells

It was previously shown that the addition of lysophosphatidic acid (LPA) to the cell culture medium resulted in the activation of PRK1 via RhoA [57, 58, 359]. As LPA binds to cell surface receptors (LPA_1 and LPA_2) and activates RhoA via the $G_{12/13}$ protein coupled to its receptors [367], there is a need to determine which element(s) in PRK1 are necessary and sufficient for controlling PRK1's response to RhoA that is activated endogenously in cells by its physiological agonist. To do this, various Myc-tagged PRK1 constructs were transiently transfected into COS-1 cells. After treating cells with physiological concentration of LPA for 30 min, cells were lysed and Myc-PRK1 was immunoprecipitated. The catalytic activity of each PRK1 construct in cells before and after LPA treatment was analyzed in an immune-complex kinase assay. Consistent with previous findings [57, 58, 359], the treatment with LPA to activate endogenous RhoA resulted in an approximately 3-fold activation of the wild-type PRK1 in COS cells, as determined in a trans-phosphorylation assay using an S6 peptide (Fig. 4.6*A*). The deletion of one to five amino acid residues from the C-terminus resulted in a progressive reduction in the activation of the PRK1-Δ946 to PRK1-Δ942 mutants by LPA. Removal of the six C-terminal amino acid residues resulted in a PRK1-Δ941 mutant that lost its capacity to be activated by RhoA (Fig. 4.6*A*), although this mutant has been shown to have a basal catalytic activity comparable to that of the wild-type PRK1 [366] and could bind GST-GTPγS-RhoA *in vitro* (Fig. 4.4). Notably, deletion of the HR1 region resulted in a significant reduction in the activation of the PRK1-ΔHR1 mutant by LPA (Fig. 4.6*B*). However, the removal of HR1 did not completely abrogate the activation of PRK1-ΔHR1 by LPA, as this mutant had a 50% increase in its catalytic activity upon treatment of LPA.

Deletion of one or three C-terminal residues from the PRK1-ΔHR1 mutant completely abolished the ability of the resultant PRK1-ΔHR1-Δ946 or PRK1-ΔHR1-Δ944 to be activated by LPA (Fig. 4.6*B*). These data suggest that the HR1 region is not the sole determinant for the activation of PRK1 by RhoA in cells. Rather, the last six amino acid residues in PRK1 play critical roles in conferring the full responsiveness of PRK1 to RhoA.

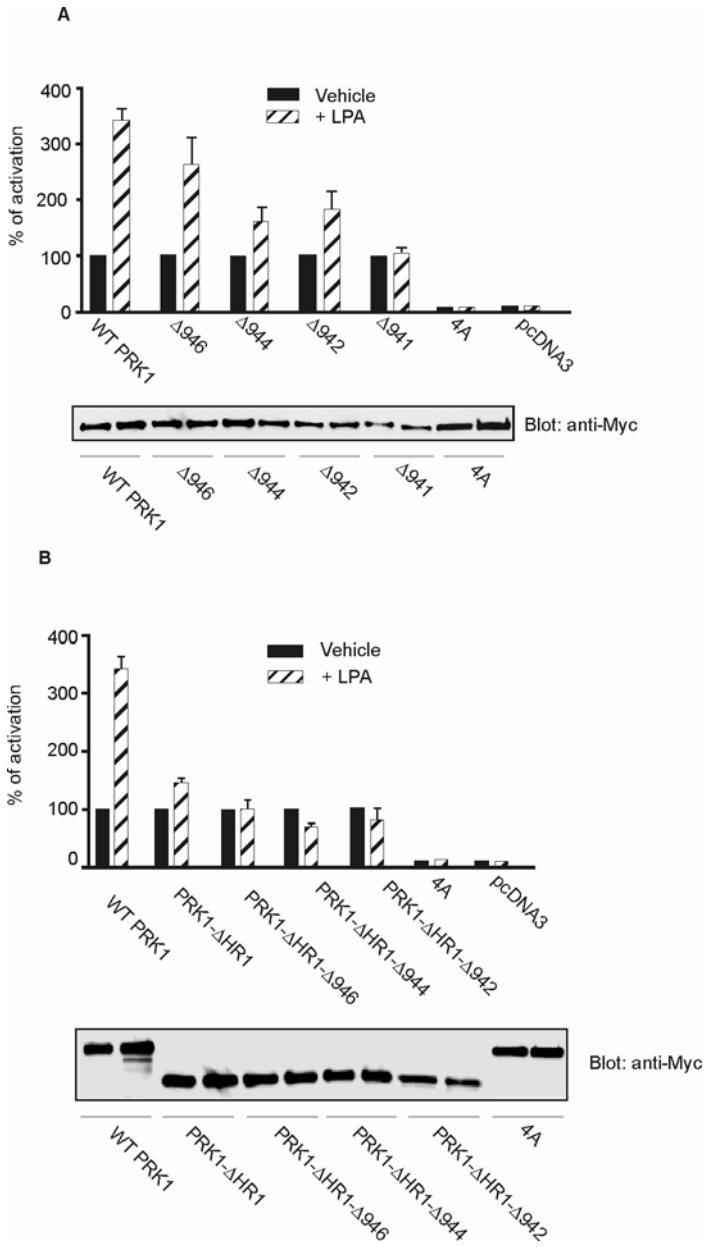


Fig. 4.6. Activation of PRK1 by LPA in cells. COS-1 cells were transfected with wild-type PRK1 or PRK1 deletion or deletion mutants as indicated. After serum starvation for 24 h, cells were treated with 5 μM LPA or vehicle for 5 min and lysed by 1% Triton X-100. The specific activity of PRK1 or its mutants was determined in an immune-complex kinase assay using an S6 peptide as a substrate. The activation of wild-type PRK1 and that of PRK1 C-terminal deletion mutants (*A*) or HR1-deletion and HR1-deletion plus C-terminal deletion mutants (*B*) was quantified after normalizing the amount of protein in each sample. Data shown are mean ± SEM of four independent experiments and are represented as percentage of phosphorylation by LPA-treated PRK1/mutant compared with that of the corresponding vehicle-treated sample. A representative Western blot used to derive the specific activity for each pair of samples is shown below the bar chart.

4.1.5 Functional Importance of the Very C-terminus of PRK1 in Medicating LPA-elicited Actin/myosin II Contractility

To evaluate the functional importance of the very C-terminus of PRK1 in transducing signals downstream of RhoA in cells, a cellular model of neurite retraction was adopted. Previous work from our lab has shown that PRK1 participates in mediating LPA-elicited neurite retraction in mouse neuronal cells N1E-115 [359]. It was established that PRK1, when transiently transfected into N1E-115 cells, signals downstream of RhoA, as co-transfection of either a dominant-negative RhoA (T19N) or a potent RhoA inhibitor botulinum ADP-ribosyltransferase C3 abolishes PRK1's ability to modulate neurite retraction upon LPA treatment [359]. In order to test which element is critical for PRK1 to mediate LPA-elicited neurite retraction, expression plasmids encoding the wild-type PRK1 or various PRK1 mutants were transiently transfected into N1E-115 cells. Once differentiated by becoming flattened and developing neurites, these neuronal cells will undergo RhoA-mediated neurite retraction and rounding within 2-3 minutes of stimulation by physiological concentration of LPA [368, 369]. As shown in Fig. 4.7*A*, the wild-type PRK1 and the PRK1-Δ942 mutant could augment LPA-stimulated neurite remodeling ($P<0.001$ compared to the vector-only control). However, the deletion of the six C-terminal residues resulted in the failure of the PRK1-Δ941 mutant in augmenting LPA-stimulated neurite remodeling, as in the cases of the HR1 deletion mutants ($P>0.05$, compared to the empty vector control). Therefore, it is demonstrated that the very C-terminus of PRK1 is not only important for the full activation of PRK1 by RhoA in cells, but is also critical for PRK1 to mediate actin/myosin II contractility downstream of RhoA in neuronal cells.

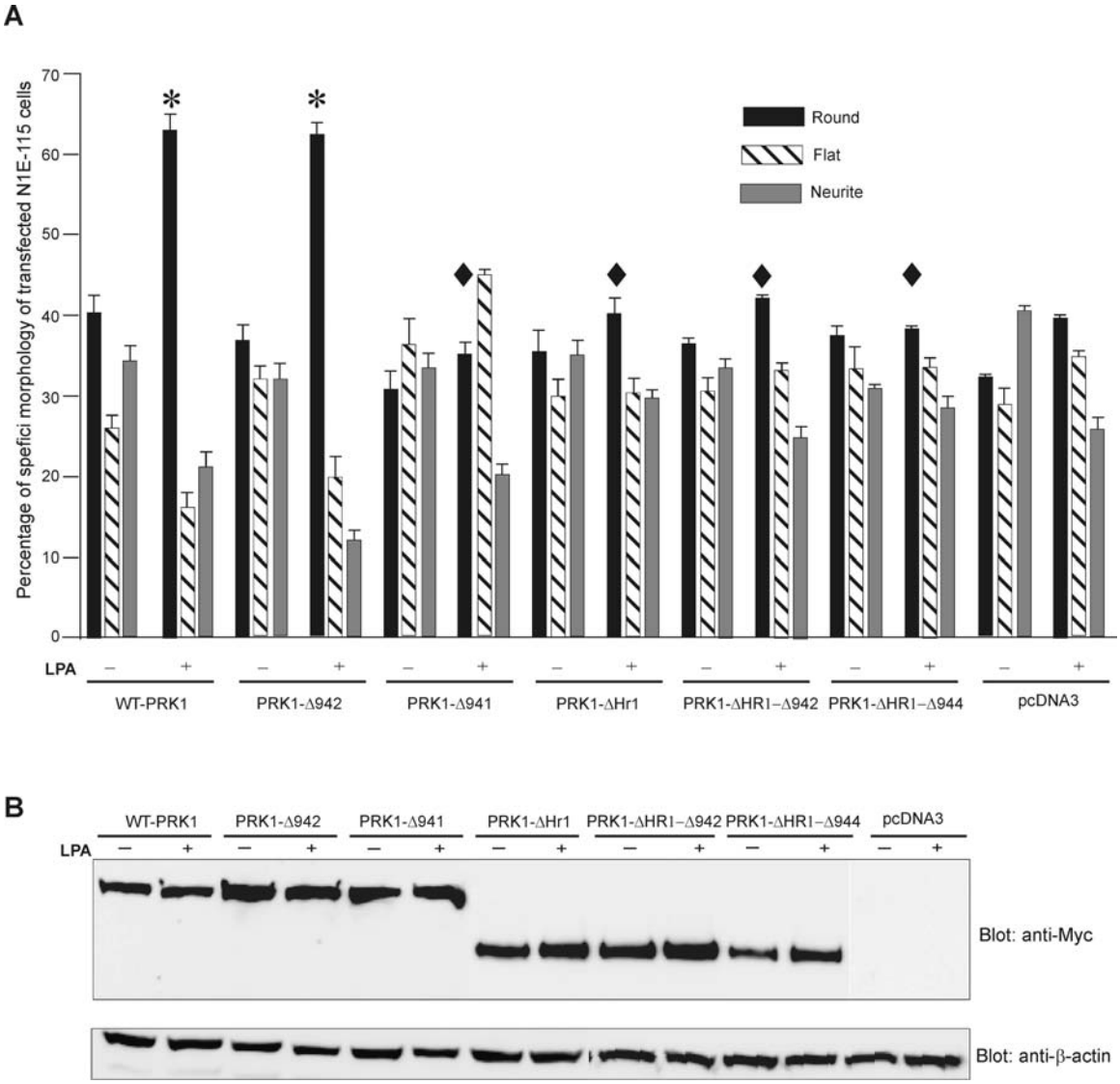


Fig. 4.7. Analysis of the capacity of PRK1 and its mutants in mediating LPA-elicited neurite retraction in neuronal cells. *A*, N1E-115 cells were transiently transfected with wild-type PRK1 or PRK1 mutants as indicated. After serum-starvation for 30 h to allow the outgrowth of neurites, cells were treated with 3 μM LPA or vehicle for 3 min and immediately photographed for morphological analysis. Data are expressed as mean ± SEM of five to six independent experiments. *B*, cells after photography were lysed using 1 x SDS-PAGE loading buffer and the total cell lysate was subjected to immunoblotting using an anti-Myc antibody to control the expression of each PRK1/mutant protein used in neurite retraction assay. A representative immunoblot showing the expression of Myc-PRK1/mutant (*top panel*) along with an immunoblot showing the content for β-actin in the cell lysate to control for the loading (*bottom panel*) are shown. *Asterisk*, $P < 0.001$ compared with empty vector control; *diamond*, $P > 0.05$ compared with vector control.

4.2 DISCUSSION

In this study, it is shown that a segment distant from the RhoA binding sites in a Rho effector serine/threonine protein kinase critically controls the activation of the kinase after the binding of RhoA and the subsequent regulation of cytoskeleton dynamics in cells is demonstrated.

Given the importance of the Rho family of small GTPases in the control of a diverse array of cellular functions, intense studies have been carried out to elucidate the molecular mechanisms of the interaction between RhoA and PRK1. The interaction between RhoA and HR1 region in the regulatory domain of PRK1 has been characterized using yeast two-hybrid system, *in vitro* binding assays either on solid support or in solution and using filter overlay assay [55-58, 268, 353, 370]. It is now established that RhoA binds with high affinity to the HR1a region of PRK1 in a GTP-dependent manner. It also interacts with the HR1b region with relatively low affinity and less dependence on the status of the nucleotide [55, 56, 353, 371]. Detailed structural analysis via X-ray crystallography and NMR suggests that both HR1a and HR1b interact with RhoA via a platform formed by an ACC fold from these two elements [266, 353]. The HR1a of PRK1 makes two possible contacts with RhoA (designated Contact I and II), while HR1b probably interacts with RhoA at one site. Although such elegant studies have provided insights into the molecular basis of RhoA-PRK1 interaction, the use of isolated domain to study protein-protein interaction is not without its limitations. For example, it was reported that recombinant protein of HR1 repeats produced in *E. coli* behaves as a dimer on gel filtration [56]. In addition, GST fusion might affect the binding of the fusion protein to RhoA due to steric hindrance as exemplified by the 16-fold reduction in affinity

between GST-Rhotekin and RhoA [371]. A more critical question that has not been addressed is whether the HR1 domain in PRK1 is sufficient for the activation of this kinase upon activation by RhoA in cells. This study attempted to answer this question by generating RhoA binding site deletion mutants followed by analyses of the RhoA binding and activation. Although it has been shown that RhoA interacts with the isolated HR1a and HR1b but not HR1c, independent studies using different methodologies have firmly established that HR1abc does bind RhoA with higher affinity in a GTP-dependent manner than either HR1a or HR1b alone [56, 371]. Therefore, an HR1 domain deletion mutant of PRK1 was generated and found to result in a substantial reduction in the amount of PRK1-ΔHR1 mutant bound to GSTγS-RhoA compared with that of wild-type PRK1 in an *in vitro* binding assay (Fig. 4.4). However, the removal of HR1 from the regulatory domain does not completely abrogate the interaction between PRK1 and RhoA, as there is still approximately 18% of GSTγS-RhoA bound to PRK1-ΔHR1. This suggests that RhoA may directly interact with PRK1 in site(s) other than the known HR1 domain. It also implies that the interaction between RhoA and its effectors is more complex than originally thought and this might involve multiple contact sites from distinct regions on the effector molecule with RhoA via a higher-order structure. Since RhoB and Rac1 are also found to bind HR1 domain of PRK1 [56, 268], the findings in this study are consistent with the recent concept that the utilization of multiple contact regions in both Rho GTPases and their effectors is one of the mechanisms underlying signal transduction of Rho in distinct cellular pathways [370].

Most of the studies regarding the role of PRK1 in medicating RhoA signaling have focused on the understanding of the nature of the physical interaction between RhoA

and PRK1. Since the pseudosubstrate segment (residues 39-53) of PRK1 that keeps matured PRK1 in an inactivated state overlaps with the HR1a ACC finger, the prevailing consensus is that the binding of RhoA to HR1a and HR1b region elicits a conformational change that dislodges the pseudosubstrate segment from the active site of PRK1 and thus activates the kinase. The structural requirement and biochemical mechanisms underlying the activation of PRK1 after the binding of RhoA are, however, poorly understood.

Earlier, it was shown that despite the fact that deletion of last seven amino acid residues from the C-terminus of PRK1 does not have a discernible adverse effect on the basal catalytic activity of this kinase, deletion of the five C-terminal residues from PRK1 completely abrogates the activation of PRK1 by arachidonic acid (see chapter 2) [366]. In the past, the C-terminal segment (the V5 domain) of PKC/PRK is thought to be too short to be considered a protein domain [340] and functionally unimportant [341]. The newly discovered critical role of the very C-terminus of PRK1 in lipid responsiveness prompted the hypothesis that the very C-terminus of PRK1 may also play a role in the activation of this kinase by RhoA. To address the critical question of whether HR1 region is necessary and sufficient for the activation of PRK1 upon binding of RhoA, the effect of deletion of HR1 or the very C-terminus on the activation of PRK1 by RhoA was studied. In an *in vitro* activation assay, it appears that GSTγS-RhoA can still activate PRK1-ΔHR1 by least 2-fold, while deletion of three to five C-terminal residues further reduces the activation of PRK1 by RhoA (Fig. 4.5). Importantly, removal of the last 6 residues from the C-terminus of PRK1 or deletion of HR1 combined with deletion of a single C-terminal residue PRK1 completely abrogates the direct activation of the mutant PRK1-ΔHR1-Δ946 by

GSTγS-RhoA. The findings of the importance of the very C-terminus of PRK1 in the activation of the kinase by RhoA *in vitro* is further supported by the data on the activation of PRK1 by RhoA in cells (Fig. 4.6). In this assay, RhoA is activated through a well established signaling pathway: from the binding of a physiological agonist of RhoA (LPA) to its G protein-coupled receptor followed by the activation of Rho guanine nucleotide exchange factors [367, 372]. In mammalian cells, the removal of HR1 does not completely abolish the activation of PRK1-ΔHR1 by LPA; however, the deletion of the last six amino acid residues at the C-terminus of PRK1 or the deletion of one C-terminal residue in PRK1-ΔHR1 completely abolishes the capacity of PRK1-Δ941 or PRK1-ΔHR1- Δ946 to be activated by LPA (Fig. 4.6). The physiological relevance of the very C-terminus of PRK1 in signal transduction of RhoA-medicated cytoskeletal dynamics is underscored by the functional analysis of PRK1 in the regulation of neurite retraction in which the PRK1-Δ941 mutant fails to augment LPA-elicited neurite retraction (Fig. 4.7).

For the other five known Rho effector serine/threonine protein kinases (p21-activated kinases, mixed-lineage kinases, Rho-kinases, citron kinase and myotonin-related Cdc42-binding kinases), the binding of GTPase causes dimerization or multimerization of the effector kinase and leads to changes to the autoinhibitory regions as well as elevated catalytic activity. The subsequent trans-autophosphorylation is thought to be responsible for releasing the autoinhibition and lead to the eventual activation of these Rho effector kinases [370]. However, such a mechanism is unlikely to be operational for PRK1, as there is no evidence for dimerization of this kinase [56]. In co-transfection systems using certain cell lines, the interaction of Rho with PRK1 seems to facilitate the phosphorylation of the

activation loop of PRK1 by PDK-1 [268, 269]. Nevertheless, the extent of phosphorylation of the activation loop of PRK1 is probably not responsible for the inability of PRK1-Δ941 to be activated by RhoA in cells, as it was previously shown that the extent of phosphorylation of the activation loop and the basal catalytic activity of this mutant are essentially the same as that of the wild-type counterpart [366].

These findings suggest that, although the very C-terminus of PRK1 is not the major site of interaction with RhoA, it critically controls the magnitude of activation after the binding of RhoA. Homology modeling and molecular dynamics simulation was employed to study the contribution of the C-terminus of PRK1 in the activation of this kinase [366]. Phe-941 and Val-942 were shown to potentially form hydrophobic interactions with Met-665 which is located in αC helix [366]. The αC helix in PKA, PKB and PKCθ and PKCι has been shown to be essential in maintaining an active kinase structure and integrates inputs from other segments in the catalytic core [85, 87, 88, 107, 111]. In addition, the extent of the twist of αC helix is known to determine the functional mode of protein kinases [338, 348]. Therefore, it is tempting to speculate that the binding of RhoA to HR1 in PRK1 elicits a series of conformational changes to displace the pseudosubstrate segment from its substrate binding site and to reposition or realign the αC helix and other elements in the catalytic domain. Such conformational changes are critical for the full activation of PRK1 and activation could not proceed in the absence of the very C-terminus, as it might hold and position the αC helix in a delicately controlled three-dimensional space required for optimal catalysis. Whether the control of the activation of PRK1 by RhoA via C-terminus residues involves an inter-molecular mechanism(s), such as

reported for Gem and Rad in the inhibition of Rho-kinases [373], awaits further experimental exploration.

Rho GTPases have been shown to be involved in the pathogenesis of cancer [372, 374]. Recently, work from this laboratory and others has implicated PRK1 in the progression of prostate cancer [359, 375]. Therefore, the development of pharmacological agents to inhibit signaling pathways downstream of specific Rho GTPases, such as RhoA, may be valuable for cancer therapy. The HR1-like domains are found in other Rho effectors, such as rhophilin [58], Rhotekin [79], Rho-kinase, citron and kinectin [371]. Targeting the HR1 region on Rho effectors may have problem in the specificity of inhibition, as there is a high sequence homology in the α2 helix of the ACC finger in HR1 where most residues in the effector involving the interaction of Rho GTPase are located. On the other hand, the very C-terminus (V5 domain) of PRK/PKC is least conserved, both in length and in amino acid sequence, among members of the PKC superfamily [340], and it is obviously not found in other Rho effectors. Thus, monoclonal antibodies, peptides or small molecules that are able to bind to the very C-terminus of PRK1 and suppress its activation by RhoA could constitute a new generation of drugs for treating diseases in which the activation of PRK1 by RhoA is implicated.

Chapter 5:

Role of the PRK2 V5 domain in kinase function

5.1 OVERVIEW

The structure of members of the PKC superfamily is highly conserved, with an N-terminal regulatory domain linked to a C-terminal catalytic domain via a linker segment (the hinge region). The catalytic core of all PKC/PRK superfamily members has 40-50% sequence identitity. The V5 domain, located at the extreme C-terminal tail is present in all PKC/PRKs, ranging from yeast to humans, but with a much lower sequence homology compared with that of the catalytic core [340]. In fact, PRK1 and PRK2 have 87% amino acid sequence identity at the kinase domain but only 46% amino acid sequence identitity at the V5 domain. In chapter 3, it was found that PDK-1 can physically interact with PRK1 at the catalytic core in the absence of the V5 domain that harbours the hydrophobic motif. In addition, the last five amino acid residues at the C-terminus of PRK1 were found to control the full lipid response of the kinase to arachidonic acid. Hence, it begs the question of whether the V5 domain plays a similar role in the regulation of PRK2.

Thus, this study aims to determine the importance of individual residues and the collective contribution of the region within the V5 domain to the catalytic activity of PRK2. At the same time, this study hoped to provide direct comparison of the mechanisms of regulation between PRK1 and PRK2, giving valuable insights to similarity and differences between these two closely related kinases

5.2 RESULTS

5.2.1 Generation and Characterization of PRK2 Mutants

A full-length human PRK2 cDNA with a Flag-epitope (GACTACAAGGACGACGATGAAAG) tag fused before the second amino acid residue was cloned into a mammalian expression vector pcDNA3 to enable identification and purification of the protein of interest. This was subsequently used as a template for the generation of point mutation and C-terminal deletion mutants. As subsequent study involved serial C-terminal deletion, the Flag tag was purposely inserted at the N-terminal. The conserved residues of the turn motif, hydrophobic motif and conserved hydrophobic residues flanking the hydrophobic motif were mutated and PRK2 was serially deleted from the C-terminal, to determine their contribution to PRK2 catalytic activity. In addition, results from PRK1 deletion and point mutants (see chapter 3) provided a working model for the generation of the deletion and point mutants which enable direct comparison of the mechanisms of regulation between PRK1 and PRK2. A schematic diagram of the PRK2 domain structure and all the PRK2 constructs used in this study is shown in Fig. 5.1.

All mutants containing deletions, point mutations and the combination thereof were generated using a polymerase chain reaction (PCR)-based mutagenesis protocol with a circular plasmid as a template as described in Materials and Methods. The deletion mutants were generated by introducing an in-frame stop codon at the designated positions, e.g. the PRK2-Δ979 mutant carried a stop codon at position 979, yielding PRK1-(1-976). **The mutants were verified by gene sequencing and the results are listed in Appendix E.**

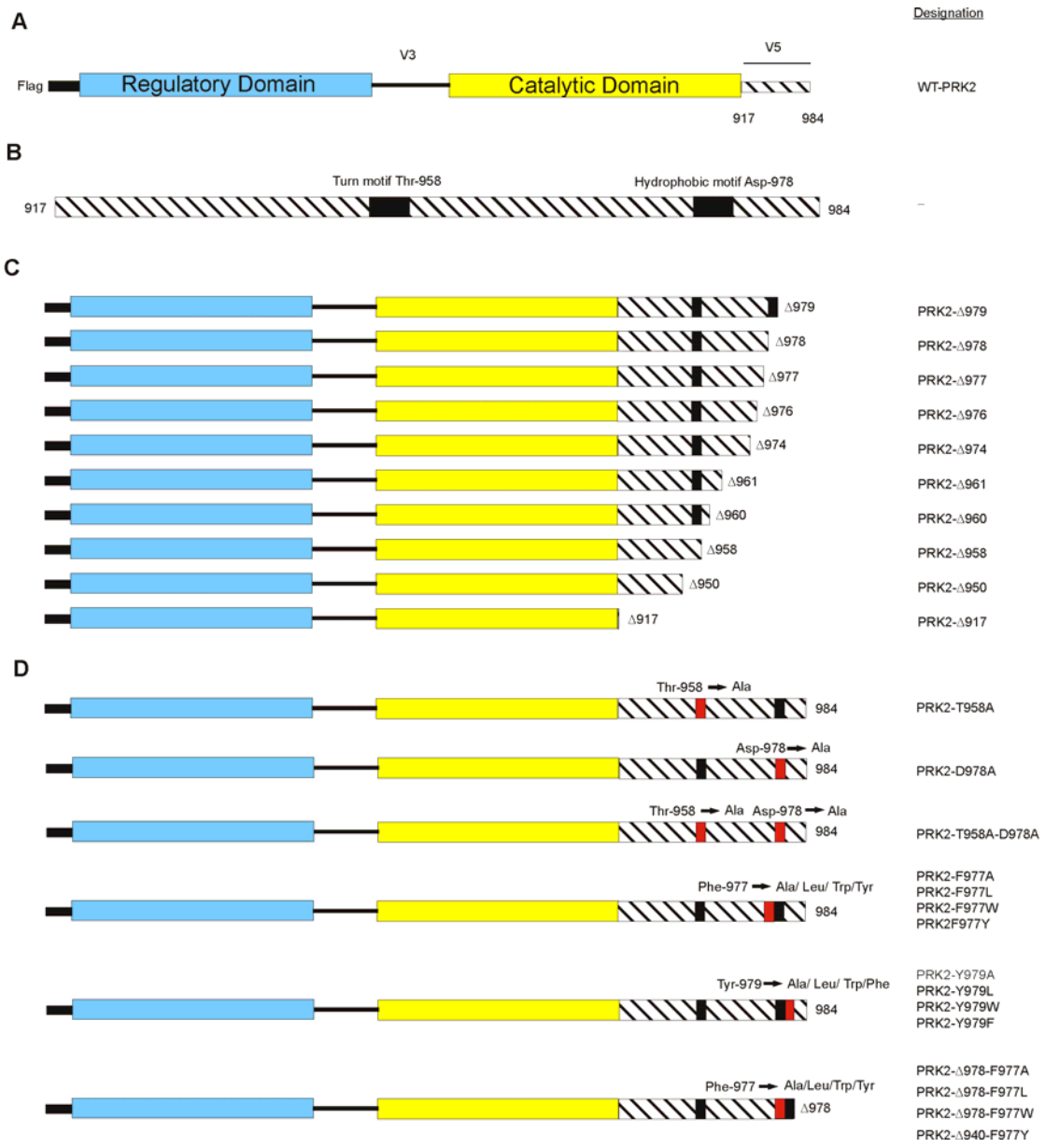


Fig. 5.1. Schematic diagram of PRK2 domain structure and PRK2 constructs used in this study. (A) Wild-type PRK2 (WT) consists of a regulatory domain (blue) and a catalytic domain (yellow) and the full length protein and mutants thereof were N-terminally tagged with a Flag epitope. The hinge region (V3) that links the two domains and the C-terminal segment (V5) are shown. Also shown are the turn motif (Thr-958) and the hydrophobic motif (Asp-978), depicted by black boxes. **(B)** Enlarged view of the V5 domain showing the turn motif and the hydrophobic motif. **(C)** Various PRK1 deletions generated. The numbers indicate the position of the stop codon, with the last expressed residue being the one just prior to the number. **(D)** Various point mutations (indicated in red) generated in full length and C-terminus deleted PRK2, e.g. T958A indicates the mutation of Thr to Alanine at position 958. The whole PRK2 molecule and the enlarged V5 domain illustrated are not drawn to scale. Nomenclatures of the constructs used are stated on the right panel.

5.2.2 The Last Eight Amino Acid Residues and the Highly Conserved Phe^{977} are Critical for the Catalytic Competence of PRK2

Although the crystal structures of the kinase domain of two PKC members were recently elucidated (PKCθ and PKCι), the extreme C-terminal of the kinase domain was too disordered to enable its structure to be assigned [87, 88]. Thus, how the V5 residues interact with the kinase domain and contribute to kinase function is unknown. To delineate the role of the V5 residues in catalytic activity, C-terminal residues were systematically deleted and the catalytic activity of these mutants was assessed by *in vitro* kinase assay. Furthermore, in PRK1, removal of the last eight amino acids resulted in a catalytically inactive kinase (Fig. 3.3A). In this section, the question of whether the corresponding segment at the C-terminus in PRK2 is also critical for catalytic function was addressed.

C-terminal residues within the V5 domain of PRK2 were deleted and the catalytic activity of these mutants was assessed in *in vitro* kinase assay. After transient transfection, the FLAG-tagged PRK2 was immunoprecipitated from COS cells lysate and was washed extensively with 0.5M lithium chloride. Lithium chloride is able to disrupt strong ionic interactions and thus was used to ensure that the purified PRK2 is free from contaminating proteins that non-specifically interact with PRK2. This purified PRK2 was subsequently subjected to an *in vitro* immune-complex kinase assay. Protamine sulfate was used as a substrate, as this arginine-rich protein binds PKC superfamily kinases and releases the pseudosubstrate and thus serves as an excellent cofactor-independent substrate of PKC/PRK [60, 200, 376].

Fig. 5.2A shows that deletion of up to seven residues from the V5 region did not significantly impair the deletion mutants PRK2-Δ979 and PRK2-Δ978. However, a significant decrease from full activity to almost null activity was observed when eight or more residues were removed from the C-terminal (PRK2-Δ977 to PRK-Δ917) (Fig. 5.2A). The full length sequence of PRK2 contains 984 residues, with the hydrophobic motif at position 978 (Asp^{978}). In full length PRK2, there are two hydrophobic residues surrounding the phosphorylation mimetic Asp^{978}: Phe^{977} and Tyr^{979}. The hydrophobic phenylalanine residue preceding the hydrophobic motif is highly conserved among the PKC members, while the succeeding hydrophobic residue is less conserved where tyrosine is replaced by phenylalanine in PRK1. The data presented thus far suggest that either the segment of eight amino acids in V5 in PRK2 was important for the catalytic activities (the removal of which results in a catalytically inactive PRK2) and/or the highly conserved hydrophobic residue preceding the hydrophobic motif Asp^{978} is critical for the catalytic competence of this kinase (absence of which results in a catalytically inactive kinase).

In PRK2-Δ978, the last residue expressed corresponded to Phe^{977}. In order to establish if the removal of Phe^{977} was responsible for this sharp decrease in catalytic activity in PRK2-Δ977 mutant, Phe^{977} was mutated in the background of PRK2-Δ978. The phenylalanine at position 977 was mutated to residues with similar aromatic side chain (tyrosine or tryptophan) or nonpolar amino acid residues with similar hydropathy index (alanine or leucine) to determine whether an amino acid with similar biochemical properties could substitute Phe^{977}. PRK2-Δ978 mutants with Phe^{977} point mutations (PRK2-Δ978+F977A, PRK2-Δ978+F977L, PRK2-Δ978+F977W, PRK2-Δ978+F977Y) were virtually inactive (Fig. 5.2B). Neither

substitution of phenylalanine by residues of similar hydropathicity or hydrophobicity was tolerated, indicating that the phenylalanine residue at position 977 in PRK2 is necessary for the kinase activity.

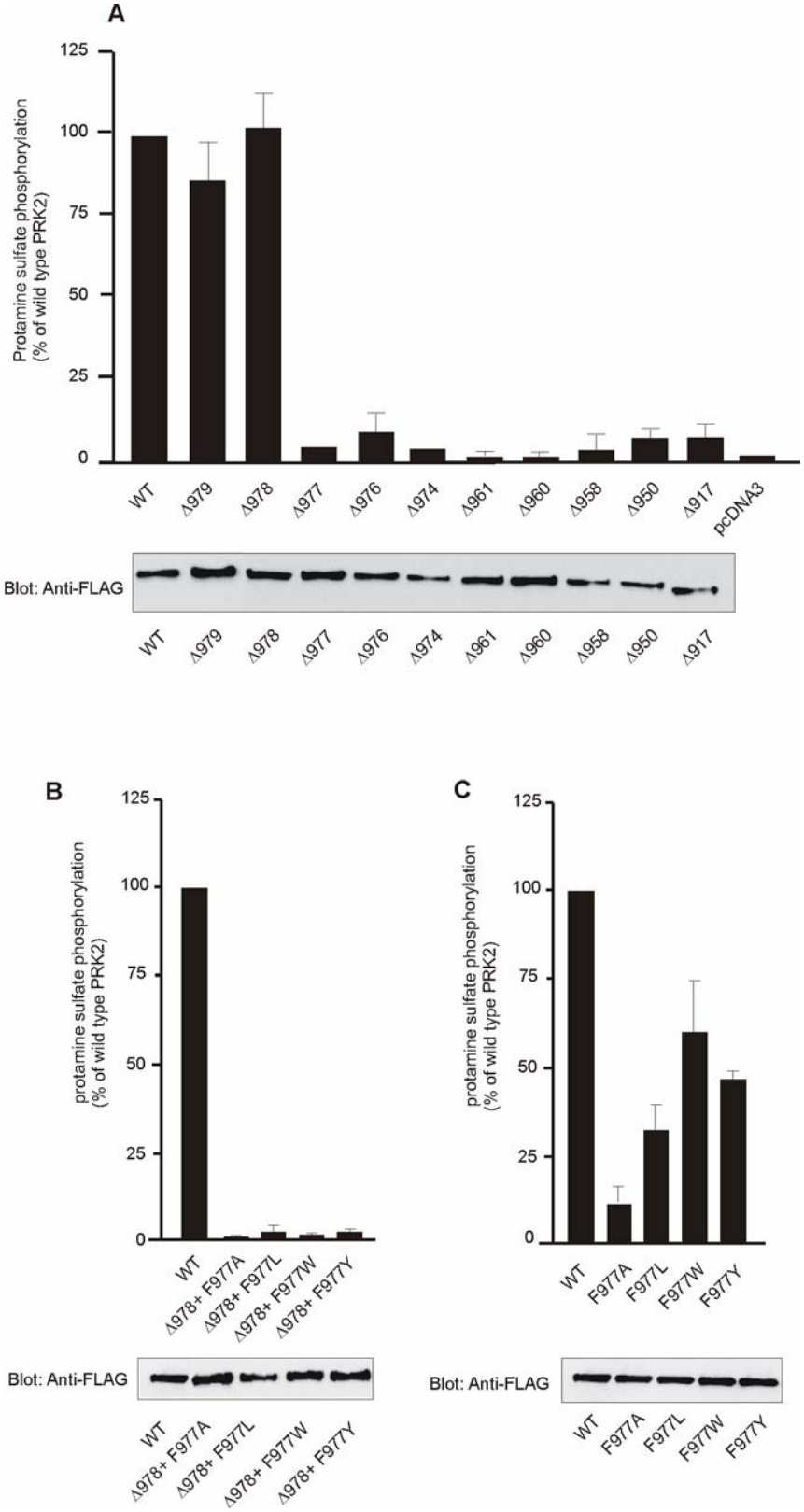
As the foregoing study was performed with deletion mutants without the extreme C-terminal, the study of point mutation of Phe^{977} in the presence of the last eight residues is necessary to examine the importance of Phe^{977} within the context of a full length PRK2. Therefore Phe^{977} was mutated to tyrosine, tryptophan, alanine or leucine in the wild-type PRK2. In the full length PRK2, mutation of the aromatic residue greatly reduced the kinase activity (PRK2-F977A, PRK2-F977L, PRK2-F977W, PRK2-F977Y) (Fig 5.2C). Mutation of Phe^{977} to residues with similar aromatic side chains (PRK2-F977W, PRK2-F977Y) resulted in kinase activities of approximately 60% and 45% respectively, while mutation to residues with similar hydrophobicity (PRK2-F977A, PRK2-F977L) resulted in 10% and 30% activity. Thus, Phe^{977} plays a critical role in catalytic function of PRK2.

However, the segment of residues at the extreme C-terminus is not functionally redundant. A comparison of the catalytic activity of PRK2 point mutation at Phe^{977} (Fig. 5.2C) and PRK2 point mutation at Phe^{977} in a background of PRK2-Δ978 (Fig. 5.2B) revealed that in the absence of the last eight amino acids, the catalytic activities of PRK2-F977A, PRK2-F977L, PRK2-F977W and PRK2-F977Y were all abolished. Therefore, the data suggest that while Phe^{977} is important for kinase activity, the segment of residues at the C-terminal is also critical to kinase function.

As mentioned earlier, there are two hydrophobic residues surrounding the phosphorylation mimetic Asp^{978}: Phe^{977} and Tyr^{979}. To determine if the conserved

hydrophobic residue Tyr^{979} succeeding the phosphorylation mimetic Asp^{978} is absolutely required and equally important as Phe^{939} for kinase activity, a Tyr^{979} point mutation was generated (PRK2-Y979A, PRK2-Y979L, PRK2-Y979W and PRK2-Y979F). Mutation of Tyr^{979} did not affect the kinase activity as much as mutation at Phe^{977} (Fig. 5.2D). Comparatively, Tyr^{979} appears to be less critical for kinase function than its upstream counterpart.

The phosphor-acceptor in the activation loop, turn and hydrophobic motif (Thr^{816}, Thr^{958} and Asp^{978}) was mutated to alanine to determine the importance of these conserved residues. The mutation of the critical activation loop residue (T816) to a non-phosphorylatable residue inhibited the activity (Fig. 5.2E), in accordance with previous studies in PKC βII, PKA and PKB [60, 93-95]. Mutation of the turn motif (T958) to alanine also resulted in a catalytically inactive PRK2 (Fig. 5.2E), in contrast to PKCα, which was reported to tolerate mutation at the turn motif [103]. The finding that PRK2-D978A has high activity (Fig. 5.2E) was expected as PRK2-Δ978 which lacked Asp^{978} also demonstrated high activity. Taken together, in PRK2, the activation loop and the turn motif were found to be more critical than the hydrophobic motif for catalytic activity.


A
Protamine sulfate phosphorylation (% of wild type PRK2)
125
100
75
50
25
0
WT
Δ979
Δ978
Δ977
Δ976
Δ974
Δ961
Δ960
Δ958
Δ950
Δ917
pcDNA3
Blot: Anti-FLAG
B
protamine sulfate phosphorylation (% of wild type PRK2)
WT
Δ978+ F977A
Δ978+ F977L
Δ978+ F977W
Δ978+ F977Y
Blot: Anti-FLAG
C
protamine sulfate phosphorylation (% of wild type PRK2)
WT
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F977W
F977Y
Blot: Anti-FLAG

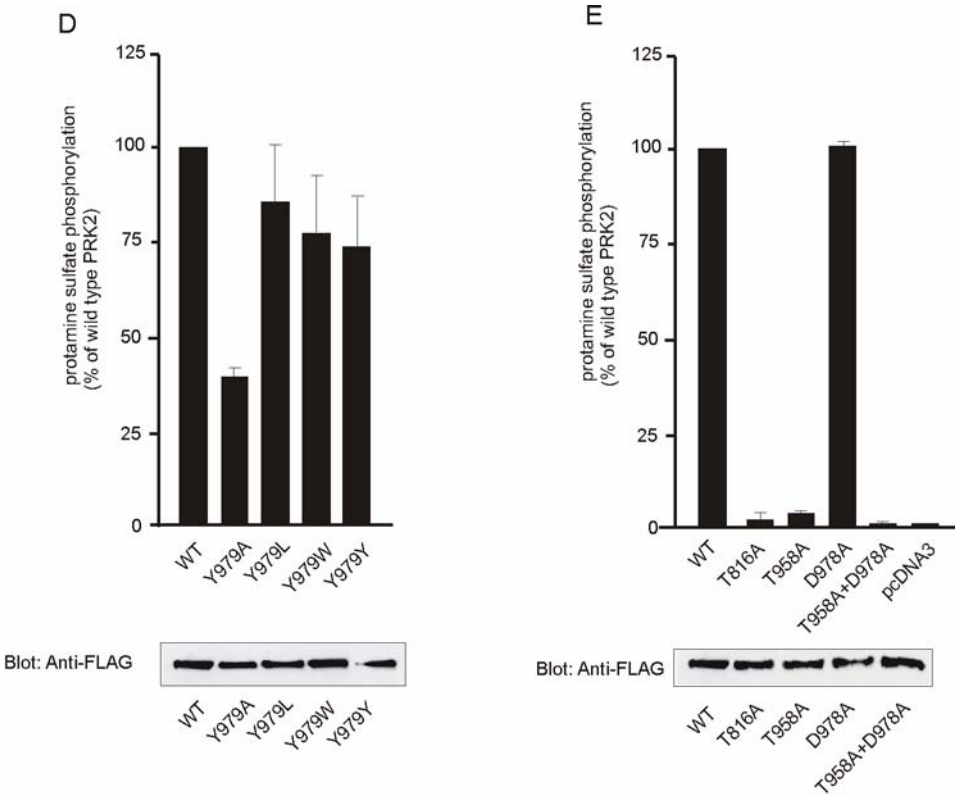

Fig. 5.2. Phe^{977} and the C-terminal of V5 domain are required for the catalytic competence of PRK2. Expression constructs encoding the wild-type or various deletion and/or point mutation mutants of PRK2 were transiently transfected into COS-1 cells. Twenty hour after transfection, the FLAG-tagged recombinant PRK2 was immunoprecipitated after lysing cells with 1% Triton X-100. **A**, the specific activity of PRK2 and deletion mutants was determined in an *in vitro* immune-complex kinase assay using 0.67 mg/ml protamine sulfate as a substrate after normalization of protein levels as described under "Materials and Methods". Results are mean ± S.E. of at least 3 independent experiments and are represented as percentage of the activity of the wild-type PRK2. **B**, the specific activity of PRK2 Phe^{977} point mutants on Δ978 background. **C,** the specific activity of PRK2 Phe^{977} point mutants. **D**, the specific activity of PRK2 Tyr^{979} point mutants. **E**, the specific activity of PRK2 activation loop, turn motif, hydrophobic motif and turn motif + hydrophobic motif mutations.

5.2.3 The C-terminal Tail of PRK2 Does Not Significantly Affect the Stability of the Kinase *in vitro*

There is a marked difference in catalytic activity between PRK2-Δ978 and PRK2-Δ977 despite the fact that they differ in only one residue (Fig. 5.2). To determine if the absence of the C-terminal residues contributed to aberrant folding of PRK2-Δ977 that rendered the mutant kinases more susceptible to degradation, the *in vitro* thermal stability of PRK2 deletion mutants was assessed. The thermal stability was studied in

conditions that closely resemble the *in vitro* kinase assay condition of 30^{o}C, except that the duration of the kinase assay was extended from 10 min to 30 min to study the effect of heat on the structural stability.

Wild-type Flag-PRK2 and Flag-PRK2 deletion mutants were transfected into COS cells. The ectopically expressed PRK2 were immunoprecipitated by anti-Flag antibody and washed with buffer containing 0.5M LiCl to remove non-specifically bound proteins before being equally divided into two aliquots, with one incubated at 30^{o}C and the other at 4^{o}C for 30 min. Both samples were subsequently subjected to an *in vitro* immune-complex assay with protamine sulfate as a substrate. After SDS-PAGE, the amount of intact PRK2 immunoprecipitated was determined using Western Blot (Fig. 5.3)

The Western blot analysis in Fig. 5.3 shows similar amounts of intact PRK2 mutants before and after heat treatment for wild-type PRK2, PRK2-Δ978 and PRK2-Δ977. To further verify that the *in vitro* stability was not compromised in PRK2 point mutants, the *in vitro* heat stability of PRK2-F977A was assessed. Mutation of Phe^{977} had no significant effect on the kinase stability *in vitro* as no significant degradation was observed (Fig. 5.3).

Thus, PRK2-Δ977 and PRK2-F977A are inherently catalytically inactive and the observed null kinase activity of PRK2-Δ977 and PRK2-F977A in Fig. 5.2 was not duo to accelerated degradation. This indicates that, at least *in vitro*, the C-terminal tail of PRK2 does not significantly affect the stability of the kinase.

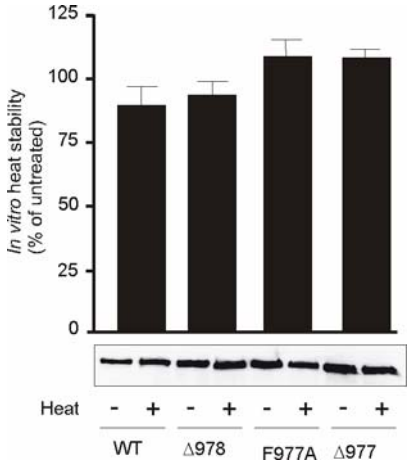

Fig. 5.3. **The V5 domain of PRK2 is not required for thermal stability *in vitro***. Flag-tagged PRK2 expression constructs were introduced into COS-1 cells. Twenty hours post-transfection, exogenously expressed PRK2 were incubated at 30^{o}C for 30 min before proceeding to a peptide phosphorylation assay. The amount of intact PRK2 construct after heat treatment is expressed as a percentage of the amount of the intact untreated sample and is shown as means ± s.e.m of three independent experiments. A representative Western Blot showing the amount of proteins before and after heat treatment is shown.

5.2.4 The Turn Motif But Not the Hydrophobic Motif in PRK2 is Necessary for Activation Loop Phosphorylation.

The first and rate limiting step in the processing of members of the PKC superfamily is the phosphorylation of the activation loop by PDK-1; isozymes that are phosphorylated at the activation loop become catalytically competent [45]. A series of studies by Alessi and co-workers provided the first evidence that PDK-1 interacts with high affinity with sequences corresponding to the hydrophobic motif by identifying the C-terminal of PRK2 in a yeast two-hybrid screen for PDK-1 binding partners [108]. This region of PRK2 was termed 'PDK-1 Interacting Fragment' (PIF), which corresponds to the 77 residues at the extreme C-terminal of PRK2.

To dissect the regions within these C-terminal residues which are required for activation loop phosphorylation, the extent of phosphorylation at the activation loop of PRK2 C-terminal deletion mutants was determined. To this end, Flag-PRK2 C-

terminal deletion mutants were expressed in COS cells. The cells were lysed and the deleted protein was immunoprecipitated with anti-Flag antibody. Following stringent washings with 1% NP-40 and 0.5M LiCl to remove non-specific interacting proteins, the immunoprecipates were separated on SDS-PAGE and analysed using Western blot with phosphor-specific antibody (P500) that only reacts with a PKC/PRK with its conserved threonine at its activation loop phosphorylated [97, 335]. The specificity of the activation loop phosphorylation under the experimental conditions employed was confirmed with the PRK2-Δ917 mutant (which had the entire V5 domain deleted and thus was unable to interact with PDK-1) whose activation loop was not phosphorylated.

Fig. 5.4 shows that PRK2-Δ978 which lacked the phosphorylation mimetic Asp^{978} and PRK2-Δ977 which lacked the conserved hydrophobic Phe^{977} were phosphorylated at the activation loop. As Phe^{977} and Asp^{978} are the main constituents of the hydrophobic motif, these results show that in PRK2; the hydrophobic motif is dispensable for activation loop phosphorylation. Indeed, this was consistent with the analysis of deletion mutants (PRK2-Δ974, PRK2-Δ961 and PRK2-Δ960) which lacked the hydrophobic motif but were phosphorylated at the activation loop (Fig. 5.4). However, PRK2 mutants without the turn motif (Thr^{958}), PRK2-Δ958, PRK2-Δ950 and PRK2-Δ917 (Fig. 5.4) were deficient in activation loop phosphorylation. Thus, the turn motif but not the hydrophobic motif is necessary for the productive phosphorylation at the activation loop of PRK2.

Fig. 5.2A shows that PRK2 lacking eight or more residues from the C-terminal were catalytically inactive. It is interesting to examine whether the extent of

phosphorylation at the activation loop is proportional to the catalytic competence of the kinase. The catalytically inactive mutants (PRK2-Δ977 to PRK2-Δ960) were phosphorylated at the activation loop (Fig. 5.2A). Therefore, there is no correlation between the extent of phosphorylation at the activation loop and the catalytic competence.

A possible explanation for the discrepancy is that C-terminal residues are important for maintaining the structure of the catalytic domain optimal for catalytic function. Although the absence of the C-terminal residues in PRK2-Δ977, PRK2-Δ974, PRK2-Δ961, and PRK2-Δ960 deletion mutants permitted phosphorylation at the activation loop, the presence of the C-terminal residues is critical for proper folding of the kinase domain to a conformation necessary for PRK2's catalytic activities.

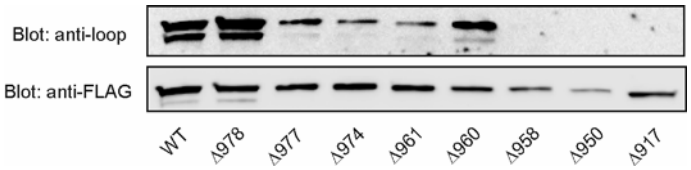

Fig. 5.4. Phosphorylation of consensus PDK-1 phosphorylation motif in the activation loop of PRK2 deletion mutants. FLAG-tagged PRK2 deletion mutants were transiently tranfected into COS-1 cells. Twenty hours post transfection, cells were lysed and FLAG-tagged PRK2 mutants were immunoprecipitated with Dynabeads coated with FLAG antibody. The immunoprecipitates were subjected to Western blot analysis using P500 anti-loop antibody specific to phosphorylated activation loop, followed by stripping and reprobing with anti-FLAG antibody. Data shown are typical of at least three independent experiments.

5.2.5 The Turn Motif and Hydrophobic Motif are Dispensable for PRK2 Interaction with PDK-1

The current dogma of PDK-1 interaction with AGC family of serine/threonine protein kinases states that PDK-1 binds with the hydrophobic motif of PKC superfamily and subsequently phosphorylates its substrate's activation loop [270]. However, Fig. 5.4

demonstrates that the turn motif but not the hydrophobic motif in PRK2 is necessary for activation loop phosphorylation as PRK2-Δ978, PRK2-Δ977, PRK2-Δ974, PRK2-Δ961 and PRK2-Δ960 were phosphorylated at the activation loop, but PRK2-Δ958, PRK2-Δ950 and PRK2-Δ917 were not phosphorylated at the activation loop (Fig. 5.4). To determine whether the failure of phosphorylation at the activation loop in PRK2-Δ958 was due to the inability of PDK-1 to interact with PRK2, the physical interaction between PRK2 deletion mutants and PDK-1 within the cell was examined.

The *in vivo* binding of PDK-1 to PRK2 was analysed in a co-transfection and co-immunoprecipitation assay in which Flag-PRK2 deletion mutants and Myc-PDK-1 were co-expressed in COS cells. After cell lysis, PRK2 mutants were immunoprecipitated via the N-terminal using an anti-Flag antibody. The immunoprecipitates were washed twice sequentially with 1% NP-40 and 300mM NaCl to remove non-specific binding. The washed immunoprecipitate was subsequently analysed in SDS-PAGE and Western blot with anti-Myc antibody to detect PDK-1 that is co-immunoprecipitated with PRK2 (Fig. 5.5, top panel). As PDK-1 and PRK2 deletion mutants have similar molecular mass, the blot was stripped of anti-Myc antibody and re-probed with Flag antibody to determine the amount of PRK2 co-immunoprecipitated (Fig. 5.5, middle panel). The similar level of expression of Myc-PDK-1 among various samples was verified by loading 20% of cell lysate in a separate SDS-PAGE followed by Western blot analysis (Fig. 5.5, last panel). The specificity of the co-immunoprecipitation was confirmed with PRK2-Δ917 deletion mutant which had its entire V5 domain removed and was thus unable to interact with PDK-1.

PRK2 was able to form stable interactions with PDK-1 without the hydrophobic motif, evident in the association of PDK-1 with PRK2-Δ978, PRK2-Δ977, PRK2-Δ976, and PRK2-Δ974 (Fig. 5.5). Contrary to the current thinking, it appears that the hydrophobic motif in PRK2 is dispensable for PDK-1 interaction, at least under the experimental conditions used in this study. This is confirmed by the fact that PRK2-Δ958 deletion mutant, in the absence of both hydrophobic and turn motif, was able to interact with PDK-1 (Fig. 5.5). The data also indicate that the failure of PRK2-Δ958 to be phosphorylated at the activation loop was not due to PDK-1's inability to interact with PRK2-Δ958. In PKCα, it was proposed that phosphorylation at the activation loop and the turn motif contributed to maintain the contact between the C-terminal (V5) domain and the catalytic core (C3/C4), thereby keeping the kinase domain in an active, phosphatase-resistant and closed conformation [103, 129]. It seems that in the partially phosphorylated state (no turn motif phosphorylation), the solitary phosphate at the activation loop was unable to maintain a closed conformation. Thus, a possible explanation for PRK2-Δ958 to exhibit PDK-1 binding but not phosphorylation at the activation loop was that the absence of a phosphorylated turn motif in PRK2-Δ958 resulted in PRK2-Δ958 deletion mutant adopting an open conformation susceptible to phosphatase action such that the phosphorylated activation loop was dephosphorylated.

A potential limitation of co-immunoprecipitation is the forced interaction of two proteins from gross over-expression of the two proteins. A feature of forced interaction of two proteins is proportionate association of the interacting protein to the amount of immunoprecipitated protein. While the amount of wild-type PRK2, PRK2-Δ958 and PRK2-Δ917 immunoprecipitated were similar (Fig. 5.5, middle panel), the amount of PDK-1 associated with wild-type PRK2 and each PRK2 deletion mutant

was different despite the equal expression of co-transfected PDK-1 among the wild-type PRK2 and PRK2 trunction mutants (Fig. 5.5, bottom panel). A higher level of PDK-1 interaction with wild-type PRK2 than PRK2-Δ958 was observed; while no PDK-1 interaction with PRK2-Δ917 was detected, indicating differential binding affinity. Thus, the positive co-immunoprecipitations observed are not artifacts due to over-expression of PDK-1.

To confirm the physical interaction between PRK2 deletion mutants and PDK-1, reciprocal detection of PRK2 association with PDK-1 immunoprecipitation is necessary. Unfortunately, reciprocal association of PRK2 with PDK-1 immunoprecipitation was not readily detected. The likely cause for this failure was the low expression level of PRK2. Without immunoprecipitation, PRK2 bands were not clearly visible in Western blots, suggesting lower expression efficiency. For unknown reasons, PRK2 expression efficiency was much lower than that for PRK1. Although using the same expression conditions were used for both PRK1 and PRK2, there was a substantial difference in expression between the two.

It is firmly established that PDK-1 functions as a master upstream kinase controlling the activation of numerous AGC kinase members [270]. Therefore, another possible reason for the failure in reciprocal binding is that PRK2 was unable to compete with other PDK-1-binding proteins in the protein-protein interactions between PDK-1 and its numerous substrates.

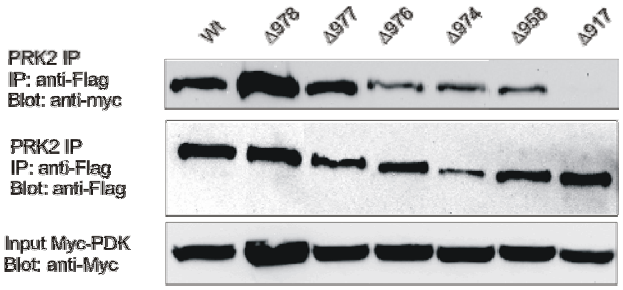

Fig. 5.5. Interaction of PRK2 and PDK in intact cells. FLAG-tagged PRK2 and Myc-tagged PDK-1 were contransfected into COS-1 cells. 24 h post transfection, cells were lysed by homogenization and immunoprecipitated with anti-FLAG coated Dynabeads. The immunoprecipitates were washed three times with buffer D consisting of 300 mM NaCl for 10 min each before being resolved by SDS-PAGE and transferred onto nitrocellulose membranes and examined by immunoblotting. The upper panel shows the amount of bound PDK-1 to the various PRK2 deletion mutants. The middle panel shows the amount of PRK2 immunoprecipitated. The bottom panel shows the amount of input of Myc-PDK-1. Data shown are representative of three independent experiments.

5.2.6 Last Seven Residues in V5 Domain of PRK2 is Required for Optimal RhoA Activation *in vivo*

The involvement of small G proteins in the control of PRK1/2 was first defined when PRK1 was found to be a target for Rho [57, 81]. The interaction of RhoA with the N-terminal HR1 domain of PRK disrupted an autoinhibitory pseudosubstrate intramolecular interaction [78], such that the incubation of PRK2 with Rho GTPase resulted in a significant increase in the kinase activity [57, 58, 82]. To determine the involvement of the C-terminal of PRK2 in the Rho GTPase regulation of PRK2 kinase activity, PRK2 C-terminal deletion mutants were tested for their kinase activity with and without RhoA.

To this end, wild-type Flag-PRK2 and Flag-PRK2 deletion mutants were transfected into COS cells. These ectopically expressed PRK2 were immunoprecipitated with anti-Flag antibody and washed with 0.5M LiCl to remove non-specific interacting proteins. To ensure that similar amount of immunoprecipitated proteins were used in

subsequent kinase assays, the immunoprecipitated PRK2 were equally divided into two aliquots. The catalytic activity of PRK2 was assayed in the presence or absence of purified GST-RhoA fusion protein in solution.

In this study, the wild-type PRK2 could not be activated by GST-RhoA (data not shown). A likely reason for the lack of activation is that the activation of PRK2 requires the GTP-bound form of RhoA. To test this hypothesis of GTP-dependency, GST-RhoA was loaded with GTP-γ-S, a GTP analogue that is not hydrolysable by GTPase. Kinase assay of the wild-type PRK2 incubated with GTP-γ-S GST-RhoA demonstrated approximately 1.8-fold increase in activity compared with that of the wild-type PRK2 without GTP-γ-S GST-RhoA (Fig. 5.6). Consistent with the results with wild-type PRK2, PRK2-Δ978 was also activated approximately 1.8-fold in the presence of GTP-γ-S GST-RhoA. The catalytically inactive deletion mutants of PRK2-Δ977, PRK2-Δ976 and PRK2-Δ958 remained catalytically inactive in the presence of GTP-γ-S GST-RhoA (Fig. 5.6). Thus, contrary to a previous report that the interaction of PRK2 with RhoA *in vitro* is nucleotide-independent [82], the activation of PRK2 by RhoA was nucleotide-dependent under the experimental conditions employed in this study.

A drawback of *in vitro* assays is that *in vitro* experimental conditions are not reflective of physiological conditions. To ensure specificity of interaction between two proteins of interest, stringent washes to remove all interacting proteins which would otherwise be interacting with either protein of interest within the cells have to be performed. Since some proteins require complexes with multiple protein-protein interactions to be functional, a study of the activation of PRK2 by RhoA in a cellular

context is necessary. This is particularly important for PRK2 since it has been found that in the presence of RhoA, there was enhanced interaction between PDK-1 and PRK2 [268].

The *in vivo* activation of PRK2 by PDK-1 was studied in a co-transfection and co-immunoprecipitation assay in which wild-type Flag-PRK2 or Flag-PRK2 deletion mutants and RhoA were coexpressed in COS cells. Two different forms of RhoA were employed in this study, a Myc-RhoA-Q61L (a constitutively active mutant which prevents intrinsic and GAP-induced GTP hydrolysis) and a RhoA-T19N (a dominant negative mutant defective in GDP exchange). RhoA-Q61L and RhoA-T19N were used as mimetics for GTP- and GDP-bound form of RhoA, respectively. After cell lysis, PRK2 mutants were immunoprecipitated using anti-Flag antibody before washing twice sequentially with 1% NP-40 and 300 mM NaCl to remove non-specific binding. It should be noted that washing with 300 mM NaCl used in the *in vivo* assay was milder than the 0.5 M LiCl used in *in vitro* assay and 300 mM NaCl had been demonstrated to allow PDK-1 binding (Section 5.1.4 and Fig. 5.5). The washed immunoprecipitate was subsequently subjected to kinase assay to determine the catalytic activity.

The wild-type PRK2 demonstrated approximately 4-fold increase in kinase activity in the presence of constitutively active RhoA-Q61L. In contrast, the same wild-type PRK2 co-transfected with dominant negative RhoA-T19N had kinase activity comparable to wild-type PRK2 without co-expression of RhoA (Fig. 5.6), suggesting that specificity of activation of PRK2 by RhoA is GTP-dependent. Furthermore, co-transfection of C3 with wild-type PRK2 or PRK2-Δ978 with RhoA-Q61L abolished

the activation of kinase activity of wild-type PRK2 or PRK2-Δ978 with RhoA-Q61L only (Fig. 5.7), thus confirming that only GTP-RhoA, but not GDP-RhoA is able to activate PRK2.

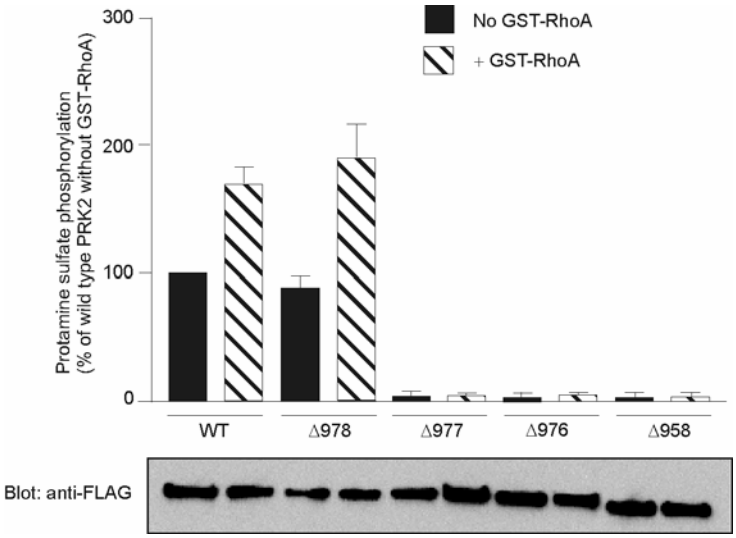

Fig. 5.6. *In vitro* activation of PRK2 by GST-GTP-γS RhoA. Expression constructs encoding the wild-type or the various deletion mutants of PRK2 as indicated were transiently transfected into COS-1 cells. Cells were lysed after 24 h and the Flag-tagged PRK2 was immunoprecipitated. The catalytic activity of wild-type PRK2 and that of the C-terminal deletion mutants was determined in the presence and absence of purified bacterially expressed GSTP-GTP-γS RhoA using an immune-complex kinase assay with protamine sulfate as substrate. Data shown are mean ± S.E. of at least three independent experiments.

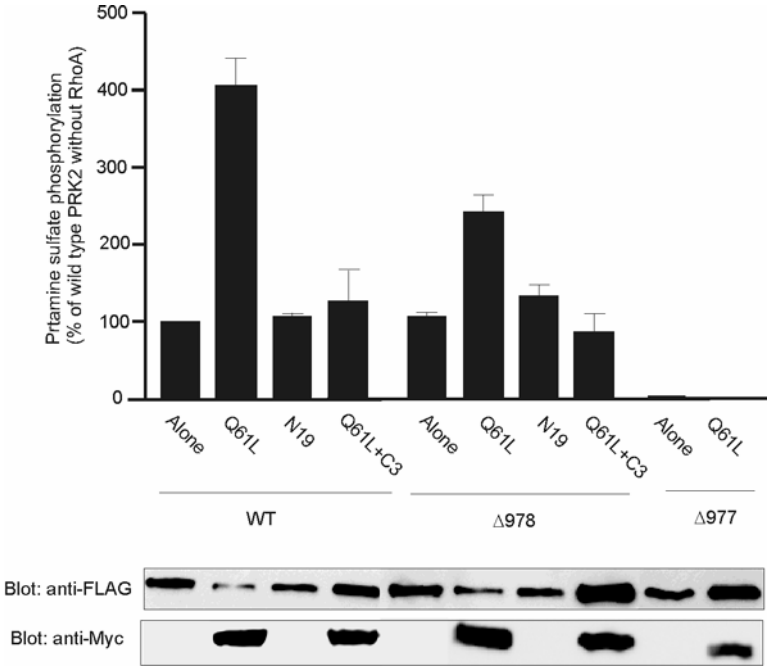

Fig. 5.7. PRK2 *in vivo* activation by RhoA. Wild-type FLAG-tagged PRK2 or PRK2 deletion mutants were co-transfected with either constitiutively active mutant RhoA (Myc-tagged Q61L) or dominant negative mutant (untagged T19N) or with potent RhoA inhibitor Botulinum ADP-ribosyltransferase C3. Twenty-four hours post tranfection, cells were lysed and the activity of the immunoprecipitated PRK2 was assayed as described in "Materials and Methods". Data are mean ± SD of at least three independent experiments.

5.2.7 The Extreme C-terminus Residues of PRK2 Negatively Regulates the Activation of the Kinase by Cardiolipin

In this study, the last seven residues in PRK2 were shown to be necessary for optimal activation by RhoA. To determine if the requirement of these last seven residues for activation of PRK2 is specific to RhoA, PRK2 deletion mutants were tested for their lipid responsiveness by analyzing autophosphorylation of PRK2 in the presence/absence of phospholipids. PRK2 were reported to show propensity for acidic phospholipids in lipid activatability. The most potent acidic phospholipid activator of PRK2, cardiolipin, has been shown to stimulate the autophosphorylation of PRK2 appreciably [264], therefore lipid responsiveness of PRK2-Δ978 to cardiolipin was examined.

The wild-type Flag-PRK2 and Flag-PRK2-Δ978 were transiently transfected in COS cells. The exogenous PRK2 were immunoprecipitated with anti-Flag antibody and washed extensively with 0.5M LiCl before being subjected to an *in vitro* kinase assay in the presence of cardiolipin. After separation of the reaction mixture using SDS-PAGE, autophosphorylation was quantified by phosphoimaging (Fig. 5.8B top panel). The protein masses of immunoprecipitated PRK2 samples present in each lipid activation assay were determined using Western Blot and were used to derive the specific activity of PRK2 (Fig. 5.8B bottom panel). Surprisingly, although wild-type PRK2 was activated by 1.5-fold, PRK2-Δ978 was activated by 3-fold by cardiolipin (Fig. 5.8A).

The seven extreme C-terminal residues were shown to be required for optimal activation of PRK2 by RhoA (Fig. 5.7). The same residues appeared to play an opposing role in cardiolipin regulation where the presence of these residues reduced activation of the kinase by cardiolipin. Thus, a novel element within the C-terminus that plays a dual role in regulation of kinase activity has been identified.

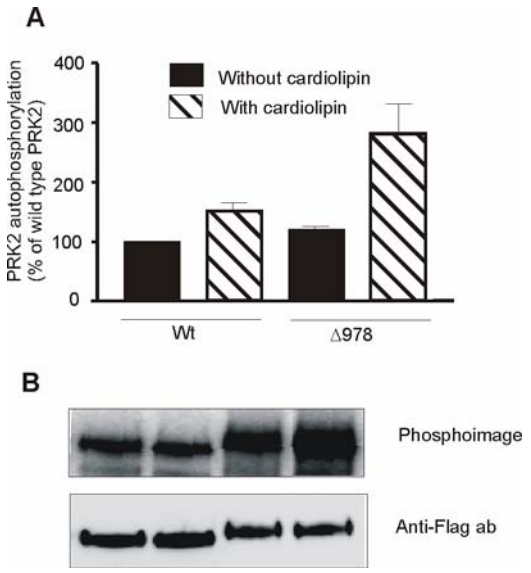

Fig. 5.8. ***In vitro*** **responsiveness of wild-type PRK2 and PRK2-Δ978**. N-terminal FLAG-tagged PRK2 were transiently transfected in COS-1 cells. Twenty-four hours post transfection, cells were lysed and exogenous PRK2 or mutant PRK2 were immunoprecipitated with anti-FLAG antibodies. The kinetic activity of PRK2 was determined in an immune-complex kinase assay with or without the activator cardiolipin (50 ug/ml final concentration). (A) The degree of autophosphorylation was determined by phosphoimaging and is expressed as a percentage to the wild-type unactivated. (B) The amount of protein immunoprecipitated was analysed by Western Blotting with anti-FLAG antibody. Data shown are representative of at least 3 independent experiments.

Appendix E:

>PRK ΔHR1_sequencing primer DWS 43

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GGGCCTTTACAACCGAAGTGGAAGCCTTAGTGGACGGAGCAGCCTCAAGGGGGAGGCAGAGAATTCCAC
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>PRK ΔHR1 Δ941_sequencing primer DWS 43

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> PRK ΔHR1 Δ944_sequencing primer DWS 68

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> PRK ΔHR1 Δ946_sequencing primer DWS 68

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Sequencing Result for PRK2

>Human PRK2 Δ979_sequencing primer DWS 68

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>Human PRK2 Δ978_sequencing primer DWS 68

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>Human PRK2 Δ977_sequencing primer DWS 68

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>Human PRK2 Δ976_sequencing primer DWS 68

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>Human PRK2 Δ974_sequencing primer DWS 68

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CAGCCTTCGACTGTGCCTTCTAGTGCCA**>Human PRK2**
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>Human PRK2 Δ960_sequencing primer DWS 68

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>Human PRK2 Δ958_sequencing primer DWS 68

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>Human PRK2 Δ950_sequencing primer DWS 68

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>Human PRK2 Δ917_sequencing primer DWS 249

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>Human PRK2 T958A_sequencing primer DWS 68

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>Human PRK2 Δ978 F977A_sequencing primer DWS 68

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5.3 DISCUSSION

The number of PKC/PRK isotypes in existence represents a great challenge to the elucidation of their function. PKC/PRKs are involved in a plethora of biological functions and interact with an assortment of proteins. Thus the difficulty of determining the individual regulation and contributions of each of the PKC isotype is immense. The V5 region was initially assigned based upon the deterioration in sequence conservation towards the end of the kinases domain [34]. This segment of 50-70 residues has been shown to play important role in PKC maturation [45] and isozyme specific functions [120, 342].

In PRK1, removal of up to 94% of the V5 domain (PRK1-Δ882, Fig. 3.2) resulted in a mutant kinase with reduced solubility (approximately 40% that of the wild-type PRK1). It was not possible to determine the contribution of the V5 domain to PRK2 solubility. Although the same expression conditions were used for both PRK1 and PRK2, for unknown reason, there was a substantial difference in expression between the two kinases. PRK2 expression efficiency was much lower than that for PRK1. A study by Palmer and Parker failed to detect PRK2 mRNA in liver tissues [43]. In the same study, the detection of relatively high levels of PRK2 in PCR analyses of myeloma compared with liver RNA suggested a higher mRNA abundance linked to the cancerous state of myelomas [43]. Thus, a possible reason for the lower expression observed in this study may be due to the high level of turnover of mRNA and/or secondary structure interference, which prevents efficient translation of PRK2 leading to a low PRK2 protein level in cells.

There is a remarkable similarity in terms of the kinase activity of C-terminus deletion mutants between PRK1 and PRK2. PRK1-Δ940 and PRK2-Δ978 both have catalytic activity as high as their respective wild-type kinases (Fig. 3.3A and Fig. 5.2A). However, further deletion of V5 residues resulted in PRK1-Δ939 and PRK2-Δ977 to be catalytically inactive (Fig. 3.3A and Fig. 5.2A). The sequence of PKA terminates at Phe^{350} which corresponds to the conserved phenylalanine residue Phe^{977} in PRK2 and Phe^{939} in PRK1. These results imply that, at least in the context of PRK1 and PRK2, either the extreme C-terminus residues are important for catalytic function and/or the presence of the conserved hydrophobic residue preceding the phosphor-acceptor is critical for kinase activity. To delineate if PRK2-Δ977 is catalytically inactive due to the absence of the conserved phenylalanine at position 977, Phe^{977} in the hydrophobic motif was mutated. The absolute requirement for phenylalanine was demonstrated in PRK2-F977A, PRK2-F977L, PRK2-F977W and PRK2-F977Y mutants. Substitution with residues similar in aromatic side chains or similar hydropathy property proved to be poor surrogates; these point mutants exhibited significantly lower activity compared to the wild-type PRK2 (Fig. 5.2C). The importance of Phe^{977} was further illustrated in a PRK2-Δ978 deletion mutant with Phe^{977} mutation. PRK2-Δ978 mutants demonstrated activity as high as wild-type PRK2, however, in PRK2-Δ978 with Phe^{977} mutation (PRK2-Δ978+F977A, PRK2-Δ978+F977L, PRK2-Δ978+F977W and PRK2-Δ978+F977Y) there was a marked decrease in kinase activity (Fig. 5.2B). Thus phenylalanine at position 977 plays a critical role in the catalytic function of PRK2.

The hydrophobic motif in PRK2 consists of $Phe^{977}Asp^{978}$ Tyr^{979}, where Asp^{978} is the phosphor-acceptor mimetic. In term of kinase activity in PRK2, the requirement for a

hydrophobic residue succeeding the phosphor-acceptor mimetic (Asp^{978}) appeared to be less strict than a need for a hydrophobic residue preceding the phosphor-acceptor mimetic. Mutation of Tyr^{979} to PRK2-Y979L, PRK2-Y979W, PRK2-Y979F and PRK2-Y979A generated kinases with higher activity than kinases with the same mutation introduced in Phe^{977} (Fig. 5.2D). In this regard, PRK2 and PRK1 are highly similar where in PRK1, mutation at Phe^{939} (corresponding to PRK2 Phe^{977}) resulted in kinases with substantial reduction in kinase activity (Fig. 3.4A,B) while mutation at Phe^{941} (corresponding to PRK2 Tyr^{978}) had much less deleterious impact on the catalytic competence of the mutant enzyme (Fig. 3.4*A, B*). The results presented here and in chapter 2 define the importance of the hydrophobic residues flanking the phosphor-acceptor site in PRK1 and PRK2. In PKCα, the hydrophobic motif was found to interact with the catalytic core to maintain an active, phosphatase-resistant, closed conformation of the kinase domain [129]. The recent elucidation of the crystal structures of PKCθ and PKCι further demonstrated that the hydrophobic motif plays a key role in stabilizing the active conformation of the kinase [87, 88].

The basis of the changes observed for PRK2 Phe^{977} and Tyr^{979} may be related to the disruption of the closed conformation consequent to the V5-C3/4 domain interactions. A working model from this study suggests that in wild type PRK2, the Phe^{977} residue appears to play a role alongside Tyr^{979} in forming an active and closed conformation of the kinase domain. Both hydrophobic residues contribute to maintain contact with the catalytic core, albeit Phe^{977} appears to play a more dominant role than Tyr^{979}. Thus in terms of kinase activity, the importance of hydrophobicity of the hydrophobic motif is defined by the phenylalanine at position 977.

The segment of residues at the extreme C-terminus of the V5 domain in PRK2 is not functionally redundant. Comparison of the catalytic activity of PRK2 Phe^{977} point mutants (Fig. 5.2C) and PRK2 Phe^{977} point mutants in a background of PRK2-Δ978 (Fig. 5.2B) demonstrated that in the absence of the last eight amino acids, the catalytic activity of PRK2-F977A, PRK2-F977L, PRK2-F977W and PRK2-F977Y were all abolished. This is consistent with PRK1 C-terminal deletion mutants where absence of the last eight residues of the V5 domain in PRK1 Phe^{939} point mutants completely abolished the kinase activity (Fig. 3.4A). Therefore, the data suggest that while the conserved hydrophobic residue in PRK1 and PRK2 are important for kinase activity, the segment of residues at the C-terminal of the V5 domain is also critical to kinase function.

A major advance in cell signaling was achieved with the purification and cloning of PDK-1 [377-380]. Subsequent studies established PDK-1 to be the upstream kinase for many key members of the AGC protein kinase superfamily. The PDK1-Interacting Fragment (PIF) which corresponds to amino acid residues 908-984 of PRK2 has been previously shown to interact directly with a pocket on the small lobe of the kinase domain of PDK1 [349]. The interaction of full length PRK2 or PIF itself [345] with PDK-1 requires the aspartic acid residue (Asp^{978}) in the hydrophobic motif, at the position equivalent to Ser-473 of PKBα. The authors proposed that the hydrophobic motifs of PRK2 are likely to be acting as PDK-1 docking sites analogous to those present in other kinases that are components of distinct kinase cascades such as mitogen-activated protein kinases, CDK2, and c-Jun N-terminal kinase [381]. As in the case of PRK1, data from this study demonstrated that the hydrophobic motif and even the turn motif in the V5 domain are not required for the interaction between

PDK-1 and PRK2 (Fig. 5.5). PRK2-Δ977, which lacks the "FDY" motif in the hydrophobic motif, was able to bind PDK-1 (Fig. 5.5) and was phosphorylated at the activation loop (Fig. 5.4). However, turn motif deletion mutant (PRK2-Δ958) was phosphorylated at the activation loop but was unable to interact with PDK-1 (PRK2-Δ958 in Fig. 5.4 and 5.5). This raises the possibility that the aromatic and acidic residues of the hydrophobic motif in PRK2 may not be the only determinants that mediate binding to PDK1. Residues N terminal of the V5 domain (without the hydrophobic region) may constitute part of the PDK1 docking site, but the strength of binding is not sufficient to support phosphorylation of the activation loop. Another possibility is that the C-terminus residues maintain PRK2 in a conformation favourable for phosphorylation of the activation loop or that they prevent dephosphorylation.

Balendran et al. reported that the mutant PRK2-D978A does not become phosphorylated at its activation loop when expressed in cells [345]. In contrast, the present study demonstrates that PRK2-D978A has activity as high as that of the wild-type PRK2 (Fig. 5.2). The two findings are not necessarily mutually exclusive. It is known that the AGC kinases require phosphorylation of the activation loop to gain catalytic competence, such that the activation loop phosphorylation site functions as a switch. Although phosphorylation at the activation loop is required for the maturation of the kinases, once this event is completed, phosphate on the activation loop is dispensable. Also, mass spectrometric analysis of the mature PKC in an over-expression system suggests that only one-half of the PKC molecules are phosphorylated at the activation loop [89]. In PRK2-D978A mutants, the fact that they have similar catalytic activity to wild-type PRK2 suggests that initially, PDK1

must bind and phosphorylate the activation loop to prime PRK2-D978A to be catalytically competent. The failure to detect a phosphorylated activation loop may be attributed to PRK2-D978A folding into a conformation that allows activation loop phosphorylation but is susceptible to dephosphorylation by phosphatases.

Furthermore, it was observed that the activity of PRK2 deletion mutants (Fig. 5.2A) did not correlate with the phosphorylation of the activation loop (Fig. 5.3). Previous studies suggest that phosphorylation of the activation loop in PRK1 and PRK2 [268, 269] activates these kinases, but finding from this study suggests that phosphorylated activation loop does not equate to catalytic activity. The results here show that even with phosphorylated activation loop, the kinase may not be fully active. At first glance, this appears contradictory to the findings with the PRK2-D978A mutant where there is no detactable phosphorylation at the activation loop but the kinase has activity as high as that of the wild-type PRK2. However these seemingly conflicting data can be reconciled based on the new findings of this study that discovered that the V5 domain plays two distinct roles in the regulation of PRK2 activity. Not only does the V5 domain increases affinity to PDK1 binding, these C-terminal residues also help to regulate the catalytic activity by folding the kinase active site in the right conformation for catalysis to take place. The basis for this seems to be in part due to the interaction of the V5 region with the kinase domain itself, inducing a closed, stable conformation. A model for this is provided by the equivalent C-terminal region of protein kinase A, which makes extensive contacts, wrapping back around the lower and upper lobes of the kinase domain [106]. In the study of phosphorylated and unphosphorylated PKB, the C-terminus was too disordered to enable its structure to be assigned, but phosphorylation-dependent changes in the core of the kinase

suggested a model in which phosphate at the hydrophobic motif orders the C helix, a key contact in the upper lobe of the kinase domain, to align residues more favourably for catalysis [107]. Thus, even though PDK-1 can interact with the N-terminal portion of the V5 domain and phosphorylate the activation loop motif, catalysis cannot take place without the critical action of the C-terminus to hold the two lobes of the kinase domain in a correct conformation.

PRK1 and PRK2 interact with activated members of the Rho GTPase family, which may activate and/or control the cellular location of these enzymes [56, 57, 82, 83, 265, 268]. Rho complexed to GTP interacts with the N-terminal regulatory region of PRK1 and PRK2, and recently the structure of Rho bound to this region of PRK1 has been solved [266]. However, controversial results have been reported regarding the binding of PRK2 with Rho family GTPase. Quilliam el al. reported that PRK2 specifically binds to RhoA in a GTP-dependent manner [261], whereas Vincent and Settleman reported that the interaction of PRK2 with RhoA *in vitro* was nucleotide-independent [82]. In the present study, GST-RhoA not loaded with GTP-γ-S could not activate wild-type PRK2 (data not shown). Instead, *in vitro* activation of PRK2 by RhoA required a GTP-bound form of RhoA (Fig. 5.6). In *in vitro* assays, GTP-γ-S GST-RhoA activated wild-type PRK2 and PRK2-Δ978 approximately 1.7 and 1.8-fold respectively (Fig. 5.7). When coexpressed with constitutively active RhoA (RhoA-Q61L), wild-type PRK2 was activated to about 4-fold; in contrast PRK2-Δ978 had approximately 2.3-fold increase in activity. Thus, the extreme C-terminus residues in the V5 domain of PRK2 may be required for optimal activation by RhoA, at least under *in vivo* conditions.

There was a significant difference between *in vitro* and *in vivo* results in the extent of activation of the wild-type PRK2 by RhoA. While the wild-type PRK2 was activated approximately 1.8-fold *in vitro*, a 4-fold increase in activity was observed *in vivo*. One reason for the observed difference in activation may be due to the presence/absence of PDK1. In the *in vitro* activation, the Flag-tagged PRK2 were immunoprecipitated and washed stringently with 0.5 M LiCl to remove contaminants. This stringent wash might have stripped PDK1 off from PRK2. In the *in vivo* system, the immunoprecipitated PRK2 were washed with 300 mM NaCl which is much milder and would not disrupt the interaction between PDK-1 and PRK2. Previously, the Rho-inactivating *Colstridium* toxin C3 transferase has been shown to inhibit lysophosphatidic acid-induced phosphorylation of PRK *in vivo* [58]. Subsequent studies established subsequently that the formation of PRK·PDK1 complex is Rho-dependent. Consistent with the requirement for RhoA in the formation of PRK2 and PDK-1 binding, the expression of *Closridium* toxin C3 transferase was found to block the PDK1 and PRK2 kinase-kinase interaction [268].

The difference in the nature of the epitope tag of RhoA employed *in vitro* and *in vivo* may be relevant to the observed difference in activation between the two studies. The GST tag of RhoA used in the *in vitro* study may have contributed to steric hindrance due to its large molecular size of 25 kDa, such that binding of GST-RhoA to PRK2 was not efficient. In contrast, the use of a ten residue Myc tag to RhoA in the *in vivo* study could circumvent the problem of steric hindrance and permit efficient RhoA binding and activation of PRK2.

Difference in post-translational modification of RhoA in the different experimental systems may also contribute to the different findings reported. Parker et al showed that *in vitro*, the HR1 domains of PRK1 and PRK2 are sufficient for binding to GTP-loaded bacterially expressed Rho, i.e. non-prenylated Rho·GTP [56]. However, for maximal PRK activation, the assembly of the ternary complex of Rho·PRK·PDK1 is necessary and the consequent PRK activation loop phosphorylation *in vivo* requires prenylation of Rho [268]. Thus, in the *in vitro* system, the GST purified RhoA was bacterially expressed and did not undergo prenylation, consequently this non-prenylated RhoA only allowed modest activation by removing the autoinhibitory region from the kinase domain. For the *in vivo* system, the expression of RhoA in a mammalian cell allows for post-translational modification, i.e. prenylation. The prenyl groups have been found to behave like "greasy handles" that can be grabbed by proteins with appropriate hydrophobic binding sites [382] (in this instance: PRK2), such that prenylated RhoA binds with a higher affinity to PRK2, thus producing a higher level of activation in the *in vivo* system.

PKC/PRKs are regulated by lipids [8] and PRK2 was reported to show propensity for acidic phospholipids in lipid activatability [264]. The most potent acidic phospholipids activator, cardiolipin, has been shown to stimulate the autophosphorylation of PRK2 appreciably [264]. In contrast to a previous study where a 7-fold increase in kinase activity was reported [264], this study was only able to detect a moderate 1.5-fold elevation in catalytic activity with cardiolipin activation (Fig. 5.8A). A possible reason for the difference in activation results between the studies is the different experimental conditions. Firstly, this study used recombinant Flag-tagged human PRK2 while Wenttenhall et al. used native purified from rat liver

PRK2 [264]. The variation in primary sequences between the human and rat PRK2 may account for, at least partly, differences in catalytic properties observed in the two independent studies. Secondly, in this study, PRK2 was immunoprecipitated using FLAG-coated beads which bind to the FLAG epitope tag at the N-terminus of PRK2. The immunoprecipitation of PRK2 via the N-terminus might have partly dislodged the N-terminal pseudosubstrate segment from the substrate binding cavity of PRK2, leading to significantly elevated basal catalytic activities and substantially reduced auto-inhibition. Therefore, this N-terminally immunoprecipitated PRK2 would have a higher basal activity that is unable to be activated by a further 4-fold after treating with cardiolipin. Similar observations were made with PRK1. In a similar experimental paradigm, the N-terminally immunoprecipitated recombinant PRK1 was activated by approximately 3-fold after the treatment with arachidonic acid (Fig. 3.8), whereas a 6-fold increase in activity was observed with native PRK1 purified from rat liver [267]. Thirdly, in this study the recombinant PRK2 was incubated for 10 min at 30^{o}C for the analysis of its catalytic activity while previous study used a 45 min reaction time [264]. The 10 min reaction time was chosen from a time course study which established that phosphorylation of PRK2 proceeded linearly within 12 min. Lastly, phosphoimaging was used in this study to determine the amount of autophosphorylation. In Wettenhall's study, the mass of PRK2 was estimated using an aliquot of PRK1 preparation by colometric method before loading to SDS-PAGE. The gel was excised and subjected to scintillation counting to determine the extent of phosphorylation. However, the actual mass of protein in the gel being excised was not determined. Thus, all these differences in experimental conditions could collectively contribute to the differences in the fold activation of PRK2 by cardiolipin between what was observed in this study and that of previously reported [264].

Surprisingly, wild-type PRK2 was activated 1.5-fold while PRK2-Δ978 was activated 3-fold (Fig. 5.8). The fact that wild-type PRK2 and PRK2-Δ978 exhibited differential activation by cardiolipin suggests negative regulation by the C-terminal residues. Thus, the last seven residues in PRK2 appear to play different roles in different signaling pathways. In RhoA signaling, this segment of residues is necessary for optimal activation while in cardiolipin signaling, the same residues negatively regulate kinase activation. While cardiolipin is unlikely to be a physiological regulator, given its exclusive location within the inner mitochondrial membrane [278], it could mimic the activation of a more physiological relevant phospholipid activator(s). Candidates include phosphatidic acid which has been implicated as a second messenger in mammalian signal transduction [278, 279, 383] and has been found to directly activate endothelial PKC [384]. The lipid responsiveness of PRK2, *in vitro*, suggests that a lipophilic second messenger may be involved in negative regulation of the kinase in cellular system.

The observation of the importance of the extreme C-terminus in PRK2 in controlling kinase function and regulating activation is consistent with recent findings in our laboratory that the very C-terminus beyond the hydrophobic motif in PKCα is essential for conferring the full catalytic competence to the kinase and for transducing signals [385]. Thus, the importance of the very C-terminus in conferring the catalytic competence in PRK2 may be a common feature among several other PKC isozymes. In conclusion, this study has identified the extreme C-terminal of seven amino acids in PRK2 is critical for the function and regulation of the kinase. The data suggest that this segment of residues in the V5 domain in necessary to maintain critical interaction

with the kinase domain to allow proper folding for catalysis. The identification of a dual role of regulation of kinase activity by the last seven amino acids suggests that the PRK2 V5 domain may be a converging point of multiple signaling pathways.

One of the goals in PKC research is to develop strategies for specific modulation of the activity and function of individual PKC isozymes. However, most of the available PKC inhibitors lack desired specificity [281, 386]. The findings of this study point to a new direction for generating PKC isozyme-specific pharmacological agents. The very C-terminus of PKC is not only the least conserved in sequence but is likely to be exposed on the surface of folded PKC as judged from the fact that most PKCs can be readily immunoprecipitated using isozyme-specific antibodies that are generated against the C-terminus. Therefore, monoclonal antibodies, peptides or small molecules that are able to interact with the very C-terminus of PKC/PRK and suppress its catalytic activity may become an invaluable tool in the study of the biology of these kinases. Moreover, such agents could represent a new generation of drugs for treating diseases in which the activation of PKC/PRKs is implicated.

General Conclusion

This project aims to investigate the role of the V5 domain in the regulation of PKC superfamily. Two members of the PKC family, PKC-Related Kinase 1 (PRK1) and PKC-Related Kinase 2 (PRK2) were used in this study to determine the following objectives:

1. the structural elements within the V5 domain of PRK1 and PRK2 that are required for the catalytic competence of the kinases;
2. the necessity of the hydrophobic motif for PDK-1 interaction and activation;
3. the function of the C-terminal extension of PRK beyond the conserved phenylalanine (Phe^{350} in PKA corresponding to Phe^{939} in PRK1 and Phe^{978} in PRK2) in the response of the kinases to lipid activators;

For the first objective, the requirement of residues within the V5 for kinase function was studied. C-terminal residues were deleted in both PRK1 and PRK2 and these mutants were tested for kinase activity. Both PRK1 and PRK2 tolerated removal of up to seven amino acids from the C-terminal (PRK1-Δ940 and PRK2-Δ978). The hydrophobic motif is Asp^{940} in PRK1 and Asp^{978} in PRK2, thus it appears that the hydrophobic motif is dispensable for PRK1 and PRK2 activity. However, with the removal of eight residues (PRK1-Δ939 and PRK2-Δ977), both mutant kinases lost their catalytic activities. Thus, the last eight amino acids in both PRK1 and PRK2 are critical for catalytic function.

For the second objective, the requirement for a hydrophobic motif for PDK-1 binding was examined. Co-immunoprecipitation studies indicate that PRK1 mutant PRK1-Δ800 in which part of the catalytic core was removed and PRK1-Δ770 where the activation loop was deleted still retained the capacity of interaction with PDK-1. As in the case of PRK1, PRK2 mutant without the hydrophobic motif and turn motif

(PRK2-Δ958) is still capable of forming stable interaction with PDK-1. Therefore, it appears that in both PRK1 and PRK2, the prevailing dogma that the hydrophobic motif functions as a substrate docking site for PDK-1 maybe an over-simplified generalization.

The relationship between PDK-1 binding followed by the phosphorylation of the activation loop in PRK1 and PRK2 and kinase activities was explored. For PRK1, phosphorylation of the activation loop closely correlated kinase activity where PRK1-Δ940 was phosphorylated at the activation loop and had activity as high as wild type kinase. On the other hand, PRK1-Δ939 was not phosphorylated at the loop motif and was catalytically inactive. However, both truncation mutants were able to interact with PDK-1. In the case of PRK2, there was no discernable pattern between the extent of phosphorylation at the activation loop and kinase activity or PDK-1 binding. An example is PRK2-Δ958 which could interact with PDK-1 but was not phosphorylated at the activation loop and was catalytically inactive. A consensus from these studies is that the interaction of PDK-1 with PRK1 and PRK2, and the productive phosphorylations of the activation loop are separate biochemical events.

For the third objective, the role of V5 domain in mediating the response to activators was analysed. In PRK1, the mode of activation by the well characterized activators arachidonic acid and RhoA was employed. The wild type PRK1 was activated three fold in the presence of arachidonic acid, however, the mutant PRK1 with its last five amino acid residues deleted could no longer be activated under the same conditions. Thus, a novel element that controls the lipid responsiveness of PRK1 at the extreme C-terminus of the V5 domain is identified. For PRK1 activation by RhoA, both *in*

vitro and *in vivo* data revealed that the removal of six residues at the C-terminus abolished the five-fold activation as seen in the wild type PRK1. Taken together, the results point to the importance of the last eight amino acid residues in the V5 domain for the full catalytic competence of PRK1. In the case of PRK2, its activation by cardiolipin and RhoA were studied. The wild-type PRK2 was activated by cardiolipin by 1.5-fold but the deletion of seven residues from the C terminal yielded a mutant PRK2 that had a 3-fold activation by cardiolipin. Therefore, it seems that the V5 domain of PRK2 performs a potential inhibitory role with regards to lipid regulation. In terms of activation by RhoA, the wild-type PRK2 could be activated 2-3 fold with RhoA, however, the PRK2 mutant lacking the last eight residues remained catalytically active.

The failure of two different activators to elevate the kinase activity in both PRK1 and PRK2 mutants lacking the last eight residues led to the hypothesis that the missing residues must affect the optimal conformation of the kinase domain such that the catalytic domain is no longer functional. Taken together, the V5 domain appears to be a determinant in isozyme-specific regulation of PRK catalytic activities.

Future Studies

To gain a more thorough understanding of the V5 domain structure-function relationship of PRK1 and PRK2, more work needs to be accomplished to examine the areas that are yet to be explored in the present study. The future research that could be pursued is briefly outlined below:

- Oligonucleotides or antibodies against the last eight amino acids in both PRK1 and PRK2 should be designed and tested *in vitro* to determine if inhibition of specific kinase can be achieved. In fact, using antibodies directed against the V5 domain in PKCζ results in low kinase activity (unpublished observation). Thus preliminary data suggests that at least in the atypical PKCs and PRKs, isozyme-specific targeting exploiting the V5 domain appears promising. A relatively new technology of protein inhibition is the use of synthetic nucleic acid ligands, called aptamers, which bind to protein targets with high specificity, affinity and with high inhibitory potential. Furthermore, these ligands are not toxic or immunogenic, thus having potential clinical relevance.

- Elucidating the three-dimensional structure is crucial to understanding how a protein in question functions. By integrating information about the function and 3D structure of proteins, more specific ways of interfering with their actions can be achieved. Thus, crystallization of PRK1 and PRK2 to determine their 3-D structure is required to completely characterize the V5 domain and allow the use of computational tools to conduct virtual docking experiments with PDK-1 and ligands targeting the extreme C-terminus.

- This study has determined that in both PRK1 and PRK2, the hydrophobic motifs are not required for PDK-1 binding. Because PDK-1 is the first and rate-limiting step, understanding how PDK-1 interacts with PRK/PKC is critical to understand the cellular controls of PKC function. Mapping the region that allows for PDK-1 binding will facilitate a deeper understanding of PRK activation. This study also found that PDK-1 binding does not correlate with the extent of phosphorylation at the activation loop of PRK. One possibility is that PDK-1 interacts with PRK *in vivo*. Thus a kinetic study using surface plasmon resonance (SPR) technology can be used to detect and monitor the biomolecular interactions.

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